Metabolic Adaptations of Pregnancy

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Metabolic Adaptations of Pregnancy

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Abstract

Pregnancy is a complex biological condition associated with profound changes in the metabolism of the mother, essential for the growth and development of the fetoplacental unit. We aimed to the study molecular pathways that contribute to the gestational alterations in lipid metabolism. The data in this report show that adaptations in lipid homeostasis during mouse pregnancy include raised hepatic cholesterol content, decreased levels of circulating cholesterol and elevated serum triglycerides. Moreover, LXR signalling contributes to the enhanced lipogenesis in early mouse pregnancy by increasing fatty acid biosynthesis in the liver. There is a gradual down-regulation of LXR targets involved in hepatic lipogenesis, cholesterol uptake and clearance following mouse placenta formation. Pharmacological activation of LXR not only blunted the reduction of these genes but also reversed the changes in hepatic and serum lipid profiles observed during normal murine pregnancy. Our results strongly suggest that LXR signalling is altered during mouse pregnancy and this is an essential adaptation to facilitate altered maternal lipid homeostasis.

Investigations were performed to establish whether maternal metabolic adaptations in energy homeostasis result from altered diurnal fluctuations in peripheral metabolic pathways. We show that pregnancy alters the activity of core clocks in liver, white adipose tissue and skeletal muscle. Early and advanced pregnancy changes the diurnal fluctuations in the expression of key metabolic genes in the liver in order to enhance or dampen lipogenesis respectively during these gestational periods. We present preliminary data suggesting that the temporal oscillations in bile acid metabolism are shifted during pregnancy independently of feeding patterns. Moreover, fatty acid homeostasis in skeletal muscle is changed during early pregnancy possibly as a consequence of the REV-ERBβ-dependent downregulation of Cpt1β-mediated lipid oxidation. Also, placenta lipid homeostasis exhibits robust temporal oscillations so that pathways mediating fatty acid and cholesterol transport as well as triglyceride hydrolysis become activated during the dark phase.

Subcutaneous and visceral white adipose tissue depots were examined to determine whether metabolic pathways in these tissues are differentially regulated during non-complicated pregnancy and gestational cholestasis. We show evidence that although both of these depots expand in the course of gestation in order accommodate triglyceride accrual, subcutaneous fat develops a pro-inflammatory phenotype whereas visceral fat remains quiescent. Feeding of pregnant mice with a cholic acid-supplemented diet raises their serum triglyceride and free fatty acid levels and reduces adipose tissue lipogenesis. Gestational cholestasis also decreases white fat inflammation in a depot-specific manner and interferes with adipose tissue remodelling and expansion. We concluded that failure of fat to grow and store surplus lipids that normally accrue during pregnancy could contribute to the development of cholestatic dyslipidaemia.

Statement of originality

All the work presented in this thesis was performed by myself unless otherwise stated in the text.

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List of Abbreviations

ABC	ATP-binding cassette transporter
ABCA1	ATP-binding cassette transporter A1
ACAT1/2	Acyl-CoA acyltransferase 1/2
ACBP	Acyl-CoA-binding protein
ACC1	Acetyl-CoA carboxylase 1
ACS	Acyl-CoA synthetase
ADRP	Adipose differentiation-related protein
AF-1	Activation function 1
AGPAT	1-acylglycerol-3-phosphate acyltransferase
AMPK	5′-AMP-activated protein kinase
ANOVA	Analysis of variance
AP2	Activating protein 2
ApoA1/B/E	Apolipoprotein A1/B/E
ARL7	Adenosine diphosphate-ribosylation factor-like 7
ARF-BP1/PAM	Adenosine diphosphate-ribosylation factor-binding protein 1 / protein associated with Myc
ATGL	Adipose triglyceride lipase
ATP	Adenine triphosphate
BAT	Brown adipose tissue
BMAL1	Brain and muscle ARNTL-like protein 1
BSA	Bovine serum albumin
BSU	Biological services unit
β-TrCP	β-transducin repeat containing protein 1
CA	Cholic acid
cAMP	Cyclic adenosine monophosphate
CAP	Catabolite activator protein
CBS	Central biomedical services
CD36	Cluster of differentiation 36
CDCA	Chenodeoxycholic acid
C/EBPα	CCAAT/enhancer-binding protein α
CETP	Cholesteryl ester transfer protein
CKIδ/ε	Casein kinases δ/ε
CLAMS	Comprehensive laboratory animal monitoring system
CLOCK	Circadian locomoter output cycles kaput
CoA	Coenzyme A
CREB	cAMP response element-binding protein
CRY1/1	Cryptochrome 1/2
CYP51A1	Lanosterol 14α-demethylase
DBD	DNA-binding domain
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulphoxide
dNTP	Deoxynucleotide
FBXL3	F-box/LRR-repeat protein 3
FGF19/21	Fibroblast growth factor 19/21
FXR	Farnesoid X receptor
DAG	1, 2-diacylglycerol

DGAT	Diacylglycerol acyltransferase
DR	Direct repeats
ERα	Estrogen receptor α
GyK	Glycerol kinase
ER	Endoplasmic reticulum
FABP3/4	Fatty acid binding proteins
FABPpm	Membrane-bound fatty acid binding protein
FAS	Fatty acid synthase
FATP1/4	Fatty acid transport protein 1/4
FDFT1	Farnesyl diphosphate farnesyl transferase 1
G3P	Glycerol 3-phosphate
GDM	Gestational diabetes mellitus
GLUT1/4	Glucose transporter 1/4
GPAT	Glycerol-3-phosphate acyltransferase
HDL	High-density lipoprotein
HFD	High-fat diet
HLF	Hepatocyte leukemia factor
HMGCR	3-hydroxy-3methylglutaryl coenzyme A reductase
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
ICP	Intrahepatic cholestasis of pregnancy
IDL	Intermediate-density lipoprotein
IDOL	Inducible degrader of LDLR
IKK	Inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells kinase
INSIG2	Insulin induced gene 2
IR	Inverted repeats
IRS-1	Insulin receptor substrate 1
IUGR	Intrauterine growth restriction
LBD	Ligand-binding domain
LBP	Ligand-binding pocket
LCPUFA	Long chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LDLR	LDL receptor
LPA	Lysophosphatidic acid
LPL	Lipoprotein lipase
LXR	Liver X receptor
LXRE	LXR response elements
MCA	Muricholic acid
MGL	Monoacylglycerol lipase
N-CoR	Nuclear receptor co-repressor
NPC1L1	Niemann-Pick C1-Like 1
NEFA	Non-esterified fatty acids
PA	Phosphatidic acid
PAP	Phosphatidic acid phosphatase
PAR	Proline- and acidic amino acid-rich
PBMC	Peripheral blood monocyte cell
PBS	Phopshate buffered saline
PEPCK	Phosphoenolpyruvate carboxykinase
PER1/2/3	Period 1/2/3

PGC1α/β	Peroxisome-proliferator-activated receptor gamma co-activator 1 α/β
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	Protein kinase A
PLTP	Phospholipid transfer protein
PPAR	Peroxisome proliferator-activated receptor
PPARE	PPAR response element
RBP4	Retinol binding protein 4
RORα/β/γ	Retinoid-related orphan receptor α/β/γ
RXR	Retinoid X receptor
SCD1	Stearoyl-coenzyme A desaturase 1
SCN	Suprachiasmatic nuclei
SDS	Sodium dodecyl phosphate
SEM	Standard error of the mean
SHP	Small heterodimeric partner
SLOS	Smith-Lemli-Opitz Syndrome
SMRT	Silencing mediator for retinoic acid and thyroid hormone receptor
SRB1	Scavenger receptor class B type I
SRC1	Steroid receptor coactivator 1
SREBP1c	Sterol regulatory element-binding transcription factor 1c
STAT1/5A/5B	Signal transducers and activators of transcription 1, 5A, 5B
TAG	Triacylglycerol
TEF	Thyrotroph embryonic factor
TEMED	Tetramethylethylenediamine
TIP47	Tail-interacting protein 47
TNFα	Tumour necrosis factor α
TRRAP	Transformation/transcription domain-associated protein
TZD	Thiazolidinedione
UCP1	Uncoupling protein 1
UDCA	Ursodeoxycholic acid
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
ZT	Zeitgeber

Chapter 1

Introduction

1.1 Overview

Pregnancy is a dynamic state involving profound changes in the hormonal milieu of the mother which then signal a myriad of adaptations in maternal nutrient metabolism. These adaptations are necessary in order to ensure a continuous supply of essential metabolites to support the growth and the development of the fetus as well as to provide the mother with sufficient energy stores to meet the demands of pregnancy and prepare for lactation (Herrera, 2002).

This introductory chapter will discuss the adaptations in lipid and carbohydrate metabolism that take place during non-complicated pregnancy. Liver X Receptor (LXR) will be reviewed as a nuclear receptor which functions as a cholesterol sensor regulating sterol clearance and *de novo* lipogenesis. Moreover, white adipose tissue will be described in its capacity as a non-homogenous endocrine organ which integrates whole-body energy storage and expenditure. Finally, the circadian clock in mammals will be detailed focusing on the transcriptional networks which govern the diurnal oscillations in key metabolic pathways.

1.2 Metabolic adaptations during pregnancy

Human pregnancy is characterised by alterations in maternal lipid metabolism which can be divided into two phases: an anabolic phase and a catabolic phase (Herrera, 2002).

1.2.1 Anabolic phase

The anabolic phase occurs in the first two trimesters of human gestation and is attributed to several factors which cooperatively increase the deposition of lipids in maternal tissues (Villar et al., 1992). The first factor is maternal hyperphagia which progressively increases throughout gestation thereby boosting the availability of exogenous metabolic substrates (Murphy and Abrams, 1993). Enhanced *de novo* lipogenesis is another factor contributing to early pregnancy anabolism. Specifically, *in situ* studies in rat periuterine adipose tissue have demonstrated a progressively increasing conversion of glucose to fatty acids and glycerol glyceride until day 20 of rat pregnancy (Palacin et al., 1991). Moreover, it has been suggested that augmented lipoprotein lipase (LPL) activity could promote fat deposition in human and rat pregnancy by hydrolysing triglyceride-rich chylomicrons and very low density lipoproteins (VLDLs) circulating in plasma, thus releasing non-esterified free fatty acids and glycerol for adipose tissue uptake (Alvarez et al., 1996, Knopp et al., 1975). Furthermore, increased intracellular utilisation of glycerol is a factor proposed to facilitate early pregnancy anabolism. In particular, under normal circumstances the conversion of glycerol to glycerol-3-phosphate, an essential precursor for triglyceride biosynthesis, is minimal due to the negligible activity of the enzyme

glycerol kinase (Thenen and Mayer, 1975). However, studies in rodents have demonstrated that glycerol kinase activity is raised under conditions of hyperinsulinaemia and exaggerated fat accumulation (Thenen and Mayer, 1975). Consequently, it has been proposed that the decreased adipose tissue lipolytic activity together with the augmented capacity of maternal tissues to employ both glucose and intracellular glycerol for the production of glycerol-3-phospate result in net triglyceride accumulation.

1.2.2 Catabolic phase

The switch to net catabolic state occurs in the third trimester of human gestation and is characterised with an accelerated breakdown of fat depots as a consequence of enhanced adipose tissue lipolytic activity (Elliott, 1975). Specifically, studies in late pregnant rats have demonstrated increased mRNA expression and activity of hormone-sensitive lipase (HSL) in white adipose tissue (Martin-Hidalgo et al., 1994). Additionally, it has been shown that the levels and the activity of adipose tissue LPL are reduced in late pregnant women (Alvarez et al., 1996) and rats (Martin-Hidalgo et al., 1994), thereby decreasing the accretion of lipids in maternal adipocytes. Non-esterified fatty acids (NEFA) and glycerol (lipolytic products released from maternal adipocytes into the circulation) are taken up by the liver where they are converted to acyl-CoA and glycerol-3-phosphate respectively and partially re-esterified for the synthesis of triglycerides; hepatic triglycerides are transferred to native VLDL particles and subsequently released into the maternal circulation. Alternatively, glycerol could be used for glucose synthesis while NEFA could be oxidised to acetyl-CoA and then utilised for energy production as well as ketone body synthesis. These pathways are essential for the fetus which during late gestation is at its maximum accretion rate and its requirements for metabolic substrates and energy fuels are greatly augmented. The preferential use of glycerol for gluconeogenesis acquires greater importance during maternal fasting periods later in pregnancy when essential gluconeogenic substrates such as alanine and pyruvate are sparse while delivery of newly-formed glucose is vital for the fetus (Zorzano et al., 1986). Moreover, studies have shown that under fed conditions in early gestation, plasma ketone body levels are lower in pregnant than in non-pregnant rats suggesting an enhanced utilisation of this energy substrate by maternal tissue as a glucose substitute (Herrera et al., 1969). Based on the fact that placental transfer of ketone bodies is highly efficient (Herrera, 2002) (ketone bodies are shown to accumulate in fetal plasma at the same rate as in maternal circulation), during periods of fasting, maternal ketogenesis becomes highly accelerated (Herrera et al., 1969) allowing the fetus to employ these molecules not only as energy fuels (Gu et al., 1992) but also as substrates for brain lipid synthesis (Edmond, 1974).

1.2.3 Hyperlipidaemia of pregnancy

Enhanced lipolytic activity in the adipose tissue of the mother in the third trimester of pregnancy precipitates the development of maternal hyperlipidaemia which mainly corresponds to increases in plasma triglyceride levels, whereas the rises in cholesterol and phospholipid concentrations are less marked (Table 1.1) (Alvarez et al., 1996). Triglycerides predominantly increase in VLDLs but they get also enriched in other lipoprotein fractions that do not normally transport them, such as low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs) (Alvarez et al., 1996). The main factors promoting the accrual of triglycerides in VLDLs during gestation are the enhanced synthesis of these lipoproteins by the liver (Wasfi et al., 1980) and their reduced removal from circulation as a consequence of the diminished LPL activity in adipose tissue (Alvarez et al., 1996). Moreover, cholesteryl ester transfer protein (CETP) is a plasma protein which facilitates that heteroexchange of triglycerides for cholesteryl esters between HDL and ApoB-containing lipoproteins (VLDL, LDL and intermediary low-density lipoprotein) (Tall, 1993). The activity of this protein peaks during the second trimester of human pregnancy and then declines in the third trimester, reaching its lowest point postpartum (Iglesias et al., 1994). It has been suggested that the changes in CETP activity correlate with the alterations in HDL-triglyceride levels which increase dramatically from the first to the second trimester of pregnancy and then rise just slightly during the third trimester before they drop postpartum. Moreover, hepatic lipase is responsible for converting buoyant triglyceride-rich HDL_2 subfraction into small triglyceride-poor HDL_3 particles and the reduced activity of this hydrolase during gestation allows for the enhanced proportional accumulation of triglyceride-rich HDL_2 lipoproteins in the plasma of pregnant women (Applebaum-Bowden et al., 1989).

Even though the molecular mechanisms contributing to gestational hypertriglyceridemia have been extensively studied, there is a substantial gap in our knowledge regarding the adaptations in the lipid metabolism of the mother which facilitate the rise in plasma total and lipoprotein-cholesterol levels during human pregnancy. Moreover, it is well established that unesterified cholesterol overload is cytotoxic and this has necessitated the evolution of complex regulatory mechanisms imposing stringent control on the intracellular levels of this sterol (Tabas, 2002) (discussed in detail in sections 1.2.4 and 1.4). However, up to date there is no information on how maternal tissues are able to limit the internalisation of the exogenous cholesterol which circulates abundantly throughout gestation. The pressing need to answer these questions is underscored by pregnancy conditions, e.g. Smith-Lemli-Opitz Syndrome (Nowaczyk et al., 2001), gestational hypercholesterolemia (Napoli et al., 1997) and hypocholesterolemia (Edison et al., 2007), where impaired cholesterol homeostasis during pregnancy has a detrimental effect on the development of the embryo *in utero* and thereby has a negative impact on its adult health.

TABLE 1.1: Plasma lipoprotein lipids in women during pregnancy and postpartum

	1st Trimester	2nd Trimester	3rd Trimester	Postpartum
Total triglycerides (mg/dL)	60 ± 5	117 ± 9	184 ± 14	81 ± 9
Total cholesterol (mg/dL)	170 ± 5	234 ± 8	254 ± 9	234 ± 9
VLDL-triglycerides (mg/dL)	22 ± 4	44 ± 6	83 ± 11	38 ± 8
VLDL-cholesterol (mg/dL)	4 ± 1	8 ± 1	18 ± 2	7 ± 1
LDL-triglycerides (mg/dL)	20 ± 2	44 ± 3	62 ± 5	28 ± 2
LDL-cholesterol (mg/dL)	89 ± 5	136 ± 8	153 ± 8	155 ± 9
HDL-triglycerides (mg/dL)	12 ± 2	26 ± 2	29 ± 2	8 ± 1
HDL-cholesterol (mg/dL)	68 ± 3	82 ± 3	71 ± 3	66 ± 3

Vales represent mean ± SE of 25 women studied throughout pregnancy and postpartum. VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. Data adapted from (Alvarez et al., 1996).

1.2.4 Transfer of metabolites from the mother to the fetus

Fetal growth is directly dependent on the availability of maternally-derived nutrients and the capacity of the placenta to transport these nutrients from the mother to fetus. The placenta is an organ anatomically configured to prevent direct contact between maternal and fetal blood and, as a consequence, relies exclusively on facilitated diffusion and active transport against concentration gradients to drive electrochemical potential and metabolite flux.

Glucose is the primary energy source for the growth of the fetoplacental unit. The high demand for this substrate in combination with the minimal contribution of fetal gluconeogenesis necessitate the development of a rapid system for transfer of maternal glucose by facilitated diffusion (via glucose transporter (GLUT) proteins) along a concentration gradient (higher maternal glucose concentrations compared to fetal drive net glucose transport toward the fetus). The GLUT family comprises 14 isoforms of integral transmembrane proteins (Mueckler and Thorens, 2013). Even though many of these isoforms have been identified in human placental tissue, GLUT1 is considered the primary glucose transporter in human placenta based on the fact that it is the only one detected as a functional protein near term in the syncytiotrophoblast (the main barrier in human placenta, composed of multinuclear cells which possess an epithelial structure comprising the microvilllous (maternal-facing) and basal (fetal-facing) membranes) (Jansson et al., 1993). GLUT1 is asymmetrically distributed across the placenta, with a three-fold higher prevalence of the transporter in the microvillous membrane than in the basal, suggesting that the rate-limiting step in transsyncytial glucose flux occurs at the basal membrane (Jansson et al., 1993). Longitudal studies have demonstrated that the overall GLUT1 expression increases during pregnancy, however, the levels of this transporter in the microvillous membrane remain unchanged during the late second and third trimesters of pregnancy whereas its basal membrane expression increases by approximately 50% over the same period (Jansson et al., 1993, Baumann et al., 2002). Moreover, it has been shown that the enhanced transport of glucose across the rate-limiting basal membrane in combination with a rise in uteroplacental and umbilical blood flow induce the substantial elevation in the supply of glucose to the fetus during the second half of pregnancy when the rate of fetal growth peaks and the demand for this nutrient is at its highest (Baumann et al., 2002).

Fatty acids play a critical role in fetal development functioning as a key energy source, essential structural components of cellular membranes and precursors for bioactive signalling compounds; fatty acids are indispensable for fetal tissue development (e.g. white adipose tissue accretion) and organogenesis (e.g. brain development). Fatty acids taken up by the placenta from maternal circulation and transported to the fetus originate predominantly from two sources: plasma NEFAs and esterified fatty acids in the form of triglycerides carried by

lipoproteins. Studies have shown that maternal triglycerides are in fact the primary source and key determinants of fetal fatty pool in humans (Elphick et al., 1978). Maternal circulating triglycerides could be taken up by the placenta via lipoprotein and scavenger receptors (Alsat et al., 1982, Furuhashi et al., 1989). Alternatively, esterified fatty acids transported in maternal lipoproteins are liberated for placental transport by hydrolysing them into NEFAs. This enzymatic step is catalysed by microvillous membrane lipases such as LPL, endothelial lipase and phospholipase A_2 (Furuhashi et al., 1989). Studies have demonstrated that placental LPL activity increases as gestation progresses and this lipase hydrolyses triglycerides in chylomicrons derived from diet as well as VLDLs (McCoy et al., 2002). On the other hand, HDL-triglycerides are a preferential substrate of endothelial lipase (McCoy et al., 2002). The expression of endothelial lipase also changes during pregnancy so that its mRNA levels are higher in term placentas than in first-trimester ones (Gauster et al., 2007). Fatty acid transport proteins (FATPs) are integral membrane proteins present in human placental membranes that mediate the preferential uptake of long chain polyunsaturated fatty acids (LCPUFA) into syncytiotrophoblast (Kazantzis and Stahl, 2012). Even though human placenta expresses five out of the six members of the FATP family (FATP1-4, 6), FATP1 and FATP4 have been most extensively studied due to the fact that their expression correlates with docosahexanoic levels in maternal plasma, cord blood and placental phospholipids, suggesting an important role of these isoforms in the transfer of LCPUFA (Larque et al., 2006a, Larque et al., 2006b). In addition to FATPs, there are two other membrane-associated proteins expressed in placenta with the ability to transport fatty acids: a placenta specific membrane bound fatty acid binding protein (FABPpm) and cluster of differentiation 36 (CD36), a fatty acid translocase (Campbell et al., 1998). Studies have shown that while FATPs and CD36 are localised on both microvillous and basal membranes suggesting that they are responsible for the transfer of NEFAs across the entire syncytium, FABPpm, which has a high affinity for LCPUFA, is exclusively located on the microvillous membrane and mediates only NEFA uptake (Campbell et al., 1997, Campbell et al., 1998). Furthermore, within the cytosol of syncytiotrophoblasts NEFAs are bound by fatty acid binding proteins (FABPs) (human placenta has been shown to express four different isoforms of FABPs (FABP1, 3, 4 and 5)) and are trafficked to cellular sites for esterification, beta-oxidation and subsequent transfer to the fetus (Lager and Powell, 2012). Finally, NEFA that cross the syncytiotrophoblast are carried to the fetal liver in α-fetoprotein where they are re-esterified and released to fetal circulation in the form of triglycerides (Herrera, 2002). Up to date, it is unclear as to which step in the process limits the rate of placental fatty acid transport to the fetus.

Cholesterol plays a key role in embryonic and fetal development. It is an essential component of cell membranes where it determines membrane fluidity and passive permeability by interacting with phospholipids and sphingolipids. This sterol also maintains cholesterol-rich microdomains,

called lipid rafts that are key for plasma membrane-dependent signalling cascades such as the sonic hedgehog pathway. Cholesterol is also a precursor for bile acids and steroid hormones (e.g. glucocorticoids which are actively synthesised in fetal adrenal during late pregnancy). Moreover, it plays important roles in cell proliferation and differentiation as well as cell-to-cell communication. Cholesterol and its oxidative derivatives, oxysterols, are regulators of various metabolic processes. It has been estimated that there must be a net accumulation of approximately 1.5-2.0 g of cholesterol for each kg of tissue that is added to the body of the developing embryo (Dietschy et al., 1993). As a consequence of its high demands for cholesterol, the developing fetus obtains this sterol either as a product of *de novo* biosynthesis or from maternally-derived deposits of cholesterol in the yolk sac and the placenta. There has been a debate as to whether human fetuses use cholesterol from the mother to support their development or whether they rely entirely their endogenously produced sterols. The most compelling line of evidence demonstrating that maternal cholesterol can be transported transplacentally to maintain the growth of the fetus comes from babies born with the congenital condition Smith-Lemli-Opitz Syndrome (SLOS) who are unable to synthesise cholesterol *de novo*. Specifically, fetuses harbouring nonsense mutations in the gene encoding Δ^7-dehydrocholesterol reductase, the enzyme catalysing the conversion of 7-dehydrocholesterol into cholesterol are capable of developing to term and are born with low levels of the sterol in their tissues (Linck et al., 2000, Nowaczyk et al., 2001). However, the exact mechanism for removal of cholesterol from maternal circulation and its delivery to the fetoplacental unit remains unclear despite the ongoing research aiming to elucidate the different stages of the process and piece them together. Specifically, is has been shown that human placental trophoblasts express LDL receptor (LDLR) (Furuhashi et al., 1989), VLDL receptor (Wittmaack et al., 1995) and scavenger receptor class B type I (SR-BI) (Wadsack et al., 2003) transmembrane proteins which mediate removal of cholesteryl esters from maternal plasma lipoproteins and their transfer to the fetal circulation. Moreover, ApoE is another possible component of the maternal–embryonal cholesterol transport system. In particular, ApoE is a ligand involved in the transport and receptor-mediated uptake of lipoproteins by various cell types and it has several isoforms that differ in their lipoprotein receptor binding affinities and consequently have profound effects on plasma cholesterol concentrations (Mahley and Rall, 2000). In humans, heterozygous mothers with an ApoE2 allele (protein isoforms defective in LDLR binding) have infants with a more severe SLOS phenotype as compared to mothers without the ApoE2 allele (Witsch-Baumgartner et al., 2004). *Ex vivo* studies in placental biopsies have demonstrated that human placenta not only expresses ApoB and microsomal triglyceride transfer protein but also is able to synthesise and secrete ApoB-100-containing lipoproteins which could mediate the transport of cholesterol from the basal membrane to the fetus (Madsen et al., 2004). *In vitro* studies in human choriocarcinoma BeWo cells cultured as a confluent monolayer have suggested that cholesterol could be taken up by the apical side of the

cells (corresponding to the trophoblasts facing the maternal circulation), transported to the basolateral surface of the cells (corresponding to the trophoblasts facing the fetal circulation) and then effluxed to human fetal serum, fetal HDL or phospholipid vesicles (Schmid et al., 2003). Also, endothelial cells from human term placentas have been shown to exhibit efficient LXR-regulated secretion of cholesterol into ApoA1 and HDL receptor particles via the ATP-binding cassette (ABC) transporters A1 and G1 (ABCA1 and ABCG1) respectively (Stefulj et al., 2009); this constitutes a potential alternative route to mediate the export of maternally-derived cholesterol from the placenta into fetal circulation. ABCA1 has also been recently identified as a gene whose variants in the mother significantly correlate with the severity of SLOS phenotype of the infant suggesting that placental cholesterol transfer pathways not only are vital for fetal development but also present as a plausible target for prenatal SLOS therapy (Lanthaler et al., 2013). Furthermore, maternally-derived cholesterol plays a role in the growth and the development also of fetuses unaffected with SLOS. Specifically, low maternal serum cholesterol levels during pregnancy are associated with reduced birthweights and a trend for raised incidence of microcephaly (Edison et al., 2007) while maternal gestational hypercholesterolemia promotes early atherogenicity by enhancing LDL oxidation and fatty streak formation in fetal aortas (Napoli et al., 1997).

Ketone bodies are essential oxidative substrates used as glucose substitutes to fuel the metabolism of both the mother and the fetus. As previously mentioned, their circulating levels in the mother are greatly increased under fasting conditions as a result of accelerated lipolysis and hepatic ketogenesis later in pregnancy (refer to 1.2.2 for details). The transfer of ketone bodies across the placenta occurs either via unfacilitated diffusion down their concentration gradient (Seeds et al., 1980) or by beta-D-hydroxybutyrate placental carrier-dependent transport (Alonso de la Torre et al., 1992). Unrestricted and rapid arrival of ketone bodies from the maternal to fetal circulation is an essential adaptation which guarantees embryonic brain development under conditions of nutrient deficiency (Adam et al., 1975). However, this adaptation could also have detrimental effects on fetal development since extended periods of maternal hyperketonaemia (as a result of gestational diabetes mellitus (GDM), starvation and high-fat intake) have been associated with increased incidence of fetal malformations, impaired neurophysiologic development as well as stillbirth (Herrera, 2002).

Amino acids play a critical role in embryonic development therefore their plasma concentrations are substantially higher in fetal than maternal circulation, indicating active transport of these peptides across the syncytiotrophoblast. Human placenta expresses over 15 different amino acid transporters and each is responsible for the uptake of several different amino acids (Jansson, 2001). Briefly, there are two amino acid transfer systems which have been recognised as essential for fetal growth: System A and System L (Jansson, 2001). System A is a sodium-

dependent accumulative transport system which facilitates the transport of small neutral amino acids such as glycine, serine and alanine into the cell (Jansson, 2001). Conversely, System L is a sodium-independent and it exchanges non-essential amino acids for predominantly essential amino acids with branched or bulky side chains, such as leucine (Jansson, 2001). Both systems are active in the microvillous and basal membranes during pregnancy (Jansson, 2001).

1.2.5 Contribution of hormones to the metabolic adaptations during pregnancy

The switch from net anabolic to net catabolic state throughout pregnancy has been attributed to alterations in the insulin sensitivity of the mother. During early pregnancy, the activity of the pancreatic beta cells is increased, as evidenced by the enhanced insulinotropic effect of glucose observed in both women and rats, while whole-body insulin sensitivity is unchanged or even augmented (Catalano et al., 1999). Consequently, it has been proposed that hyperinsulineamia in the first two trimesters of gestation is the principal factor promoting maternal lipogenesis and fat deposition. In contrast, the last trimester of gestation is associated with progressive insulin resistance which is believed to cause the increase in adipose tissue lipolysis, hepatic gluconeogenesis and ketogenesis while inhibiting the activity of LPL in adipose tissue (Catalano et al., 1999).

Moreover, estrogen is a reproductive hormone which increases progressively throughout pregnancy and is key factor contributing to the development of maternal hyperlipidaemia as a means to ensure reproductive success by enhancing the availability of lipoprotein-derived cholesterol and free fatty acids to the ovaries and placenta in order support steroidogenesis as well as fetal development. Specifically, it has been shown that estrogen enhances the production of light VLDLs and also reduces the expression and activity of hepatic lipase in the liver thereby inhibiting the clearance of circulating triglyceride-rich lipoproteins (Applebaum-Bowden et al., 1989). Moreover, studies in postmenopausal women undergoing hormone replacement therapies have demonstrated that exogenously administered estrogens increase the levels of plasma HDL-cholesterol and triglycerides and reduce the concentrations of total cholesterol and LDL-cholesterol (Godsland, 2001). Also, estrogen is shown to increase insulin receptor binding in primary rat adipocytes thereby possibly enhancing insulin sensitivity during pregnancy (Ryan and Enns, 1988).

Progesterone is another key reproductive hormone whose levels escalate throughout gestation, although it exerts no significant effect on lipoprotein metabolism. Studies in premenopausal women administered with the progestogen-only pill have shown marginal reductions in the levels of circulating total cholesterol and triglycerides (Barkfeldt et al., 2001). Decreasing trends are detected also in the plasma concentrations of HDL-cholesterol while LDL-cholesterol

remains unchanged (Barkfeldt et al., 2001). However, it has been suggested that administration of progesterone derivatives is able to blunt the changes in serum lipid profiles due to estrogen (Sitruk-Ware, 2000). The magnitude of these effects varies depending on the androgenic properties of the different progesterone derivatives; natural progesterone is not able to significantly modify estrogen-induced adaptations in lipoprotein profiles (Sitruk-Ware, 2000). Also, *in vitro* studies have demonstrated that progesterone is able to induce insulin resistance by decreasing insulin binding to cultured rat adipocytes (Ryan and Enns, 1988).

Furthermore, several placental hormones have been implicated in re-programming maternal physiology in order to achieve an insulin resistant state. Human placental lactogen increases 30-fold throughout gestation and is shown to stimulate insulin secretion in human islets (Brelje et al., 1993). Moreover, studies in cultured rat adipocytes have suggested that while this hormone has no effect on insulin receptor binding, it is able to interfere with post-binding glucose transport thereby promoting insulin resistance (Ryan and Enns, 1988).

Human placental growth hormone is a peptide which differs from pituitary growth hormone by 13 amino acids and has also been implicated in promoting insulin resistance in late gestation. It is detectable in plasma from week five and its levels progressively rise throughout gestation effectively replacing pituitary growth hormone in the maternal circulation from mid-gestation onwards (Wu et al., 2003). *In vitro* studies have shown that this trophoblast hormone is able to elicit insulin secretion in cultured human islets (Brelje et al., 1993). Moreover, transgenic mice overexpressing the human placental growth hormone gene become larger than their normal littermates and are hyperinsulineamic as well as insulin resistant (Barbour et al., 2002).

Adipose tissue-secreted factors such as leptin and adiponectin have also been considered as candidates actively mediating insulin resistance of pregnancy (reviewed in detail in 1.5.7 and 1.5.10) (Barbour et al., 2007). For instance, tumour necrosis factor alpha (TNFα) is a cytokine produced by various tissues including white adipose tissue (Zhang et al., 1994) and placenta (Chen et al., 1991). There is an inverse correlation between the insulin sensitivity of women at different stages of pregnancy and the plasma concentrations of this pro-inflammatory factor, moderately decreasing in early gestation and then rising progressively until term (Kirwan et al., 2002). Moreover, TNFα is recognised as a predictive marker of insulin resistance and its plasma levels are higher during advanced gestation in women with GDM than in women with non-complicated pregnancies (Cseh et al., 2000). *Ex vivo* studies have also demonstrated that placentas from women with GDM secrete higher amounts of this cytokine than control placentas (Coughlan et al., 2001). Furthermore, *in vitro* studies have shown that TNFα downregulates insulin receptor signalling in cultured adipocytes (Hotamisligil et al., 1994), myocytes (del Aguila et al., 1999) and hepatocytes (Feinstein et al., 1993) by increasing serine

phosphorylation of insulin receptor substrate 1 (IRS-1) and diminishing insulin receptor tyrosine kinase activity (Rui et al., 2001). In pregnancy, there is evidence of impaired insulin receptor and IRS-1 tyrosine phosphorylation (Friedman et al., 1999) as well as increased serine phosphorylation in skeletal muscle (Shao et al., 2000) indicating that TNFα could be a key hormonal factor mediating insulin resistance in human gestation.

These data strongly suggest that the metabolic adaptations throughout pregnancy result from the dynamic alterations not only in the absolute plasma concentrations of hormones but also in the quantitative balance of these peptides relative to each other. Consequently, maternal circulating factors which have opposing effects when acting individually (e.g. impact of estrogen and progesterone on insulin sensitivity) in fact work synergistically to promote the shift to insulin resistance in late gestation and supply the developing fetus with glucose.

1.2.6 Alterations in maternal lipid homeostasis and their impact on fetal growth

As was previously discussed, hyperlipidaemia is a pivotal gestational adaptation; it is necessary to ensure sufficient and continuous supply of glucose and lipids to support the development of the fetus. However, failure to maintain the circulating levels of plasma lipids and lipoproteins within physiological ranges during pregnancy results in maternal dyslipidaemia and associated abnormal fetal growth.

Specifically, intrauterine growth restriction (IUGR) is a pregnancy condition where the fetus does not reach its predetermined growth potential (small-for-gestational-age fetuses). One of the causes of IUGR is preeclamptic placental dysfunction where insufficient remodelling of the spiral arteries during placentation results in inadequate blood flow to the fetoplacental unit and consequently limited supply of nutrients to the fetus despite their availability in the maternal circulation (Khong et al., 1986). However, it has been shown that women with pregnancies complicated by IUGR but without clinical symptoms of pre-eclampsia have significantly reduced levels of cholesterol, LDL-cholesterol, total $VLDL_2$, intermediate-density lipoprotein (IDL) and LDL as compared to matched pregnant controls (Sattar et al., 1999). These findings strongly suggest that failure to develop gestational hyperlipidaemia in the mother compromises the supply of oxidative and lipogenic substrates to the fetus and causes IUGR. Moreover, it has been demonstrated that small-for-gestational-age fetuses have higher risk of developing impaired glucose tolerance and diabetes as well as cardiovascular diseases including hypertension and stroke later in life (Neitzke et al., 2011).

On the other hand, gestational pathologies such as GDM and obesity, where maternal plasma levels of circulating lipids are supraphysiologically raised, are associated with fetal overgrowth

(large-for-gestational age infants). Specifically, obese pregnant women present with significantly increased levels of circulating triglycerides in combination with reduced HDL-cholesterol levels and hyperglycaemia as a consequence of exacerbated peripheral insulin resistance in the third trimester of gestation (Ramsay et al., 2002). As a result, transplacental delivery of nutrients is quantitatively and qualitatively altered leading to the birth of infants increased neonatal fat mass and body fat percentage (Sewell et al., 2006). Moreover, offspring of women with obesity and/or GDM are not only prone to adverse side effects at birth as a consequence of macrosomia, e.g. fetal death, prematurity, birth trauma, and respiratory distress syndrome, but also have a high risk of developing obesity, impaired glucose tolerance, and type 2 diabetes in adulthood (King, 2006, Yessoufou and Moutairou, 2011).

1.3 Intrahepatic cholestasis of pregnancy

Intrahepatic cholestasis of pregnancy (ICP) is the most common pregnancy-specific liver disease affecting between 0.2% and 2% of all pregnancies (incidence reports vary depending on ethnicity and geographical location) (Williamson and Geenes, 2014). It classically presents after 30 weeks of pregnancy and is generally asymptomatic except for the development of pruritus (itch) typically on the palms of the hands and the soles of the feet (Williamson and Geenes, 2014). Women with ICP present with significantly raised serum bile acids (above 14 μM) and impaired liver function (Laatikainen and Ikonen, 1977). ICP spontaneously resolves following placenta delivery.

1.3.1 Aetiology of ICP

The aetiology of ICP is multifactorial, with susceptibility conferred by both endocrine and genetic factors. Specifically, evidence of the genetic component to the disease come from pedigree studies demonstrating that siblings have a 17-fold increased risk of developing the disease (Eloranta et al., 2001). Genetic variation in *ABCB4, ABCB11, NR1H4, ATP8B1* and *ABCC2* have been reported in ICP (Williamson and Geenes, 2014). However, these genetic variants accounts only for a small proportion of cases and many individuals develop ICP without harbouring known genetic susceptibility. Also, women carrying variation in these genes do not necessarily develop ICP and most individuals that develop ICP are asymptomatic outside of pregnancy, suggesting that gestation unmasks cholestasis in predisposed women. At present, the primary events that expose women to risk of developing pregnancy-induced cholestasis are not known.

There is evidence for a hormonal aspect to the aetiology of ICP. Women with previous history of ICP are shown to develop cyclical itching outside of pregnancy (Lammert et al., 2000) and

cholestatic symptoms have also been reported in women following the administration of oral contraceptives (Williamson et al., 2004). In addition, the disease is more common in twin than singleton pregnancies (Gonzalez et al., 1989) and most women develop ICP in the third trimester when reproductive hormones are at their highest. Furthermore, it has been demonstrated that administration of the depot estrogen, ethinylestradiol propanolsulphonate, to 20 healthy women significantly increased the total serum bile acid concentration (Barth et al., 2003). Delivery of estradiol-17β-D-glucuronide causes not only the endocytotic internalisation of bile salt export pump (ABCB11) in a multidrug resistance-associated protein 2 (ABCC2) dependent pathway but also reduces the canalicular accumulation of bile salts in rats (Stieger et al., 2000). In rat livers, estradiol decreases the expression of the Na^+-taurocholate-cotransporting polypeptide which mediates the removal of bile acids from sinusoidal blood (Simon et al., 2004) and ethinylestradiol reduces the mRNA levels of organic anion transporters (Geier et al., 2003) underscoring the contribution of estrogens to the pathophysiology of ICP. On the other hand, progesterone has also been implicated in the development of this disease. Administration of natural progestins early in pregnancy increases the incidence of ICP (Bacq et al., 1995). Also, recent studies from our group have shown that epiallopregnanolone sulphate is supraphysiologically raised in the serum of ICP patients and that this progesterone metabolite functions as a partial agonist to the farnesoid X receptor (FXR), inhibiting FXR-mediated bile acid efflux and fibroblast growth factor 19 (FGF19) secretion (Abu-Hayyeh et al., 2013).

1.3.2 Association between ICP, GDM and gestational dyslipidaemia

Recent studies have reported increased incidence of GDM following the onset of ICP, suggesting that the impairment in bile acid homeostasis associated with the disease could disturb glucose metabolism, preventing efficient glucose excursion following an oral glucose challenge (Martineau et al., 2015, Wikstrom Shemer et al., 2013). Moreover, women with ICP also present with dyslipidaemia characterised with significantly raised fasting levels of plasma total triglycerides as well as total and LDL-cholesterol as compared to matched pregnant controls; circulating levels of HDL-cholesterol are significantly reduced in these women (Dann et al., 2006, Martineau et al., 2015). However, it is not clear how ICP causes these derangements in the lipid profiles of women affected with the disease. There is mounting evidence in other metabolic diseases such as diabetes and obesity that the activity and homeostasis of white adipose tissue is impaired and this plays a key role in the precipitating dyslipidaemic phenotype of these diseases (Hajer et al., 2008). Part of this project aims to determine how white adipose tissue is affected by pregnancy cholestasis and whether it contributes to the development of gestational dyslipidaemia.

1.3.3 Impact of ICP on fetal health

ICP is associated with an increased incidence of adverse perinatal outcomes, including spontaneous preterm delivery, meconium staining of the amniotic fluid, fetal asphyxia, respiratory distress and stillbirth (Williamson and Geenes, 2014). It has been proposed that elevated serum bile acids levels in the fetal compartment interfere with the transplacental gradient responsible for the removal of bile acids away from fetoplacental unit, thereby promoting the accumulation of these toxic molecules in fetal circulation and meconium (Brites, 2002). Additionally, it has been proposed that ICP has a positive effect on fetal growth and as a consequence there is increased incidence of large-for-gestational-age infants in pregnancies complicated with ICP, but in the absence of GDM or pre-eclampsia (Wikstrom Shemer et al., 2013). Recent studies from our group have also demonstrated that maternal ICP has a long-term effect on the health of the offspring by programming increased predisposition to metabolic disease (Papacleovoulou et al., 2013). In more detail, it was observed that the 16-year-old offspring of women whose pregnancies were complicated by ICP (without GDM) reported sex-specific differences in body mass index, fat distribution, cholesterol, and insulin resistance (Papacleovoulou et al., 2013). Similarly, mice exposed to increased bile acid levels *in utero* develop hepatosteatosis in combination with a severe obese and diabetic phenotype following a Western diet challenge (Papacleovoulou et al., 2013). Moreover, as previously mentioned, GDM increases the risk of the offspring of developing diabetes, obesity, and metabolic syndrome in later life (Yessoufou and Moutairou, 2011, Hajer et al., 2008). Given the higher incidence of maternal impaired glucose tolerance observed in pregnancies complicated by ICP, this may provide an additional explanation for the metabolic changes observed in the offspring of affected women.

1.3.4 Pharmacological treatment of ICP

ICP is commonly treated with ursodeoxycholic acid (UDCA) which is present in trace amounts in normal human serum. Randomised controlled human trials have reported that administration of UDCA reduces total serum bile acid levels in maternal and fetal umbilical cord sera (Glantz et al., 2005). UDCA is also shown to significantly improve liver function tests, pruritus and fetal outcomes (Glantz et al., 2005) as well as placental morphology and function (Geenes et al., 2011, Serrano et al., 1998). Even though the exact mechanism of action is not clearly understood, it has been proposed that UDCA, which is a hydrophilic bile acid, could decrease the hydrophobicty of the bile acid pool making it less hepatotoxic and facilitating its excretion (Lucangioli et al., 2009, Zollner et al., 2003).

1.4 Cholesterol metabolism

Cholesterol is a vital lipid molecule which functions as an essential constituent of all eukaryotic plasma membranes and also serves as the sole precursor for the biosynthesis steroid hormones, bile acids, vitamin D and certain signalling compounds such as oxysterols (also reviewed in section 1.2.4). Therefore, all pathways involved in the accumulation and clearance of this sterol are stringently regulated in order to ensure constant intracellular cholesterol homeostasis.

1.4.1 Cholesterol biosynthesis

Although nearly all mammalian cells contain the enzymatic machinery necessary to generate cholesterol *de novo*, the liver accounts for 70% of the total biosynthesis of the sterol in the body while the intestine supplies 20% (Dietschy et al., 1993). Intracellularly, cholesterol is synthesised in the endoplasmic reticulum from acetyl coenzyme A (acetyl-CoA) via the mevalonate pathway (Goldstein and Brown, 1990); 3-hydroxy-3methylglutaryl coenzyme A reductase (HMGCR) is an enzyme which catalyses the rate-limiting step of this pathway, namely the conversion of 3-hydroxy-3methylglutaryl coenzyme A to mevalonic acid (Rodwell et al., 1976). Following its synthesis, cholesterol together with the majority of its intermediate precursors such as lathosterol and desmosterol, leave the endoplasmic reticulum (ER) in order to maintain low sterol levels in the organelle. The newly generated sterol is then transported via non-vesicular mechanisms to the plasma membrane where it is incorporated into lipid rafts or is taken up by circulating acceptor particles; a proportion of the synthesised sterol also gets targeted to other intracellular compartments, e.g. endosomes (Baumann et al., 2005). Cytosolic free cholesterol is also converted into cholesteryl esters (reaction catalysed primarily by the enzyme acyl-CoA acyltransferase 1 (ACAT1) (Chang et al., 1997)), more hydrophobic derivatives of the sterol, which are then stored in lipid droplets. At the cellular level, it is essential to maintain the pool of free cholesterol reduced to a minimum in order to avoid cytotoxicity and apoptosis. It has been established that oversaturation of the plasma membrane, as well as any other organelle lipid bilayers, with cholesterol results in reduced permeability and fluidity which has an adverse effect on certain integral membrane proteins which require conformational freedom for proper function, and also limits non-facilitated diffusion (Tabas, 2002). Moreover, accumulation of unesterified cholesterol leads to the formation of cholesterol crystals which could damage the cell by physically disrupting the integrity of intracellular structures (Tabas, 2002). Excess free sterol accumulation can also promote the oxidation of cholesterol to oxysterols, some of which may be cytotoxic (Tabas, 2002).

1.4.2 Cholesterol absorption and influx

Aside from *de novo* synthesis, mammalians obtain significant amounts cholesterol from intestinal absorption. Cholesterol present in the intestinal lumen derives from several sources, including diet, bile, intestinal secretion and desquamated epithelial cells (van der Wulp et al., 2013). Biliary cholesterol is unesterified and could be directly taken up by the enterocytes, whereas dietary cholesterol needs to be released from food and therefore undergoes emulsification, hydrolysis and micellar solubilisation prior to absorption (Figure 1.1) (van der Wulp et al., 2013). In the enterocytes, cholesterol is transferred to the endoplasmic reticulum where it is esterified by the action of ACAT2 (Lee et al., 2000). The majority of the esterified cholesterol, together with triglycerides, phospholipids, ApoB-48, ApoC2 and ApoE, is incorporated into chylomicrons which move through the lymphatic system to enter the blood stream and reach the liver (Nelson and Cox, 2013b). In addition, a proportion of the enterocytic cholesterol is packaged into HDL particles which then carry the sterol directly to the liver (Brunham et al., 2006a). Circulating chylomicrons are depleted from triglycerides as a consequence of LPL-mediated hydrolysis of lipids in the capillary beds of peripheral tissues such as adipose tissue, skeletal muscle and heart. The depleted chylomicron remnants are then taken up by the hepatocytes via ApoE-receptor-mediated endocytosis and where they are subjected to lysosomal degradation in order to release the cholesterol component (Nelson and Cox, 2013b).

Hepatocytes package excess cholesterol and cholesteryl esters, together with triglycerides and phospholipids, into ApoB-100- and ApoE-containing VLDL particles which are then effluxed into the circulation (Nelson and Cox, 2013b). The triglyceride content of lipoproteins is continuously exhausted via the action of LPL in extrahepatic tissues thereby converting the VLDLs into LDLs which are rich in cholesterol and cholesteryl esters. Peripheral cells take up LDLs via the LDLR and thereby expand their intracellular cholesterol pool.

1.4.3 Cholesterol efflux and clearance

Cholesterol removal is imperative for cholesterol homeostasis: it serves to prevent over-accumulation of the sterol in the cells and subsequent cytotoxicity. Since extrahepatic cells are not able to catabolise it, excess cholesterol is packaged into HDL particles and transferred to the liver for reutilisation and excretion, a process termed reverse cholesterol transport (Figure 1.1).

Hepatic cells are also limited in their ability to dispose of cholesterol as they do not possess enzymes capable of degrading the steroid nucleus of this molecule. Instead, they have evolved

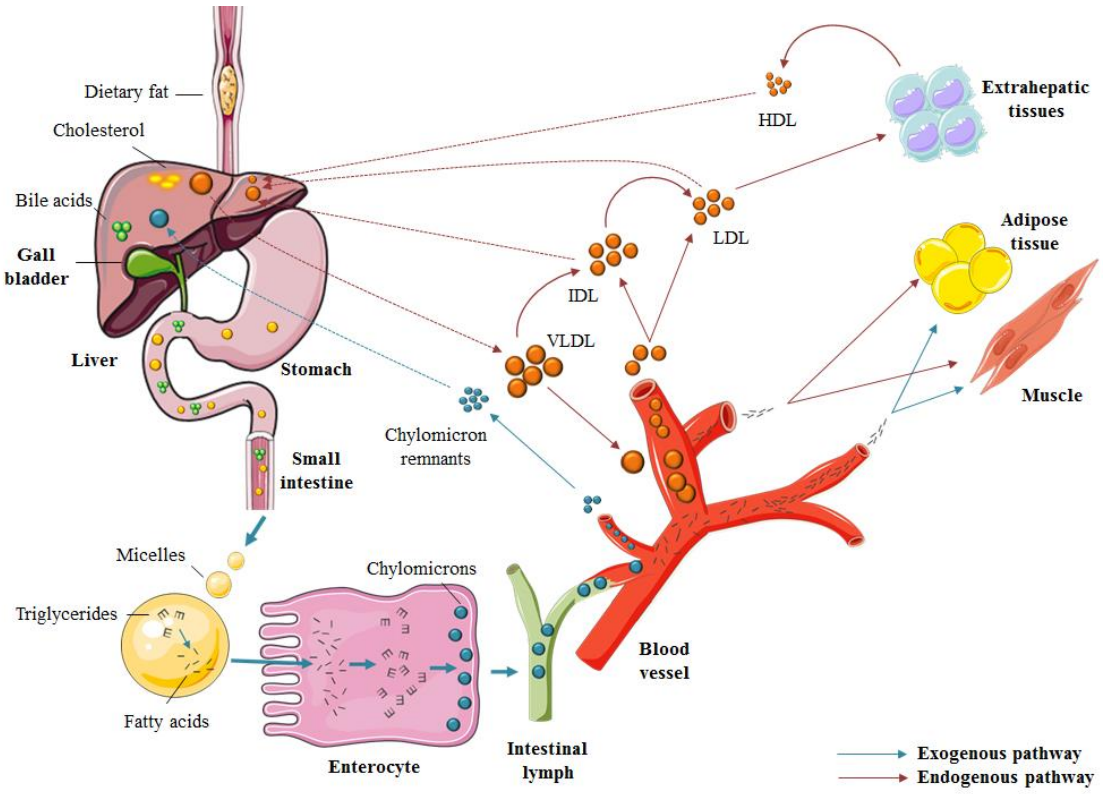


FIGURE 1.1: Cholesterol and triglyceride transport pathways. Dietary fats get emulsified by bile acids (amphipathic compounds synthesised from cholesterol in the liver, then stored in the gall bladder and released upon the ingestion of a fatty meal) in the small intestine and form mixed micelles. Intestinal lipases degrade dietary triglycerides and free fatty acids get released. Luminal non-esterified fatty acids and free cholesterol are taken up by intestinal enterocytes. In the enterocytes, fatty acids are re-esterified to triglycerides and together with cholesterol get incorporated into chylomicrons. Chylomicrons are released from the intestinal mucosa into the lymphatic system and then enter the blood, which delivers them to peripheral organs (adipose tissue and muscle). In tissue capillaries, extracellular lipoprotein lipase hydrolyses triglycerides to fatty acids and glycerol, which are utilised by cells in the target organs; liver utilises cholesterol-rich chylomicron remnants. In the liver, excess triglycerides and cholesterol get packaged into very-low density lipoprotein (VLDL) particles and releases them into the blood. In tissue capillaries, LPL hydrolyses VLDL to intermediate density lipoproteins (IDLs) and then to low-density lipoproteins (LDLs); liberated fatty acids get utilised by peripheral organs. Cholesterol-rich LDL particles are either utilised by peripheral tissues or transported back to the liver. Extrahepatic tissues incorporate excess cholesterol into high-density lipoprotein (HDL) particles which then carry the sterol back to the liver for biliary excretion.

an intricate series of reactions that modify cholesterol by the addition of hydroxyl groups and the shortening of the conjugated side-chain to synthesise bile acids. Although peripheral tissues express some of the enzymes involved in this pathway, liver is the only organ which expresses the full complement of seventeen enzymes which mediate bile acid synthesis. The conversion of cholesterol to the primary bile acids, cholic acid (CA), and chenodeoxycholic acid (CDCA), occurs via either the neutral (also known as classic) or acidic (also known as alternate or mitochondrial) pathway. In the neutral pathway, the first and rate-limiting step is catalysed by the microsomal cholesterol 7α-hydroxylase (CYP7A1) which mediates hydroxylation of cholesterol at the 7^{th} position. By contrast, the acidic pathway is initiated by hydroxylation of cholesterol at the 27^{th} position by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1), which then generates a substrate for oxysterol 7α-hydroxylase. The acidic pathway preferentially produces CDCA, whereas the neutral pathway produces both CA and CDCA, the relative levels of which are regulated by the 12α-hydroxylase enzyme (CYP8B1). Primary bile acids can be further modified by intestinal bacteria to secondary bile acids. Also, 95% of all bile acids undergo glycine or taurine conjugation and these modifications are pivotal because they not only increase the solubility if these molecules but also prohibit their free movements across cell membranes (Thomas et al., 2008).

Bile acids are secreted from the hepatocytes into the bile canaliculi where they get mixed with cholesterol and phospholipids, and eventually get transported into the gall bladder. Following the consumption of food, enteroendocrine cells localised in the lining of the duodenum secrete the hormone cholecystokinin which promotes contraction of smooth muscle cells of the gall bladder and relaxation of the sphincter of Oddi, resulting in the pulsatile secretion of bile into the duodenum. Within the lumen of the duodenum the bile salt-containing mixed micelles facilitate the digestion of dietary lipids and the absorption of fat-soluble vitamins. Once they have completed their emulsification role, 95% of bile salts are reabsorbed in the distal ileum and are transported back to the liver where they become reconjugated and resecreted into the bile canaliculi; this process of bile acid turnover is called enterohepatic circulation. Bile acids which are not taken up by the enterocytes are subsequently excreted via the faeces (Thomas et al., 2008).

In addition to biliary excretion, cholesterol can be removed from the body via transintestinal efflux. Specifically, recent studies have suggested that enterocytes could take up cholesterol from circulating lipoproteins and then secrete it apically into the intestinal lumen for faecal clearance (Kruit et al., 2005, Temel et al., 2010). Although the exact molecular mechanisms contributing to this pathway remain elusive, it has been shown that transintestinal cholesterol efflux is operative in human intestine and therefore could function as an alternative route for cholesterol excretion (Le May et al., 2013).

Overall, cholesterol is a pivotal molecule whose biosynthesis, influx and clearance are stringently regulated. Liver X receptor is a member of the nuclear receptor superfamily of ligand-activated transcription factors which plays a key role in the regulation of every domain of cholesterol metabolism (there are other molecular mechanisms involved in the control of neutral sterol homeostasis, however, they are outside the scope of the work presented in this document).

1.4.4 Nuclear receptor superfamily

Nuclear receptors are a family of transcriptional factors which regulate cognate gene networks and thereby control whole-body physiology and metabolism (Evans, 1988). They are able to detect hormonal (e.g. estrogen), metabolic (e.g. oxysterols) and nutritional (e.g. fatty acids) cues and then translate them into alterations in gene expression. In humans, this family comprises 48 proteins all of which have a conserved modular structure organised into four major functional domains (Figure 1.2) (Aranda and Pascual, 2001). Namely, they have an N-terminal domain which is highly variable (both amino acid sequence and size) and intrinsically unfolded, and which harbours the transcriptional activation function 1 (AF-1) region (Figure 1.2). The DNA-binding domain (DBD) is approximately 70 amino acids long and is highly conserved in both sequence and structure. The DBD consists of two zinc-finger modules which target the receptor to specific DNA sequences known as hormone response elements. The hinge region is positioned C-terminally to the DBD and it allows for the free rotation of the DBD. The hinge region is poorly conserved and in many cases it hosts nuclear localisation signals in addition to amino acid residues that undergo post-translational modifications that modulate the activity of the transcription factor. The C-terminal section of the nuclear receptor encompasses that ligand-binding domain (LBD) which harbours the activation function 2 (AF-2) region. In simple terms, the LBD is responsible for "ligand recognition" and it ensures the selectivity and specificity of physiologic responses. In practice, crystallography studies have revealed that the LBD is a conserved core of 12 α-helices and a short two-stranded anti-parallel β-sheet which are structured into a three-layered sandwich fold generating a hydrophobic ligand-binding pocket (LBP) (Bourguet et al., 2000). The size and the shape of these pockets vary substantially between the different receptors and could be further modified by molecules, unrelated to the endogenous ligands, which bind away from the LBP (allosteric regulation). It has been demonstrated that ligand-binding is a process which depends on the dynamic interaction between the agonist molecule and the LBP and results in shifts in the conformation of α-helix 12 (H12) (Bourguet et al., 2000, Li et al., 2003). Upon binding of an agonist, H12 adopts a stable active position so that, together with helices 3 and 4, it forms a hydrophobic groove designed to recognise LxxLL motifs present on coactivators. Following their association with the LBD of the receptor, the coactivators induce first the recruitment of acetyltransferases which allow the establishment of a permissive chromatin environment and then recruitment of the

basal transcriptional machinery which mediates target gene expression. In contrast, binding of an antagonist prevents the positioning of H12 in an active conformation and redirects it to the coactivator binding site where it forms a block, precluding cofactor association. Furthermore, partial agonists/antagonists are a third class of nuclear receptor ligands which are not able to stabilise the H12 conformation and therefore the activity of these molecules depends on the relative abundance of coactivators and corepressors in the cell (Nahoum et al., 2007). The fourth class of nuclear receptor ligands are called inverse agonists and they encompass peptides that bind to the LBD and stabilises the interaction with corepressors (le Maire et al., 2010, Bourguet et al., 2010).

LXR is a member of the nuclear receptor superfamily and shares the same modular organisation with conserved structural domains (Figure 1.2) (Apfel et al., 1994, Willy et al., 1995).

1.4.5 Liver X receptor

LXR was discovered after a screening of a rat liver cDNA library and was initially classified as an orphan nuclear receptor because its natural ligands were unknown (Apfel et al., 1994, Willy et al., 1995); this receptor has been "adopted" by several physiological ligands since then. Specifically, it has been demonstrated that LXR functions as an intracellular "cholesterol sensor" since oxidised derivatives of cholesterol (oxysterols) are specific agonists for this receptor (Lehmann et al., 1997). Oxysterols which are most potent in LXR activation include: 24(S),25-epoxycholesterol, which is produced in hepatocytes and macrophages, 24(S)-hydroxycholesterol, an abundant cholesterol metabolite in brain tissue and 22(R)-hydroxycholesterol, an intermediate in steroid hormone production; neither free cholesterol nor cholesteryl esters are physiological LXR ligands (Lehmann et al., 1997). LXR has two isoforms, LXRα (NR1H3) and LXRβ (NR1H2), which are encoded by different genes. Even though these two receptors are highly homologous, sharing approximately 78% identity in the amino acid sequence of their DBDs and LBDs, they have distinctive patterns of tissue distribution: LXRα is expressed in tissues with high metabolic activity such as liver, intestine, adrenal glands, adipose, macrophages, lung, and kidney (Apfel et al., 1994, Willy et al., 1995) whereas LXRβ is ubiquitously expressed (Seol et al., 1995, Song et al., 1994). LXR constitutively binds to isoforms of the retinoid X receptor (RXR), a nuclear receptor activated by 9-cis retinoic acid, forming a permissive heterodimer which could be activated by either receptor's ligands (Janowski et al., 1996). The LXR/RXR heterodimer regulates gene expression through binding to specific DNA sequences i.e. the LXR response elements (LXREs) in the promoter region of the LXR target genes. LXRE comprises two direct repeats (DR) of the consensus sequence AGGTCA separated by four nucleotides (DR-4) (Teboul et al., 1995, Willy et al., 1995). However, studies have shown that LXR transactivation could be also mediated through inverted

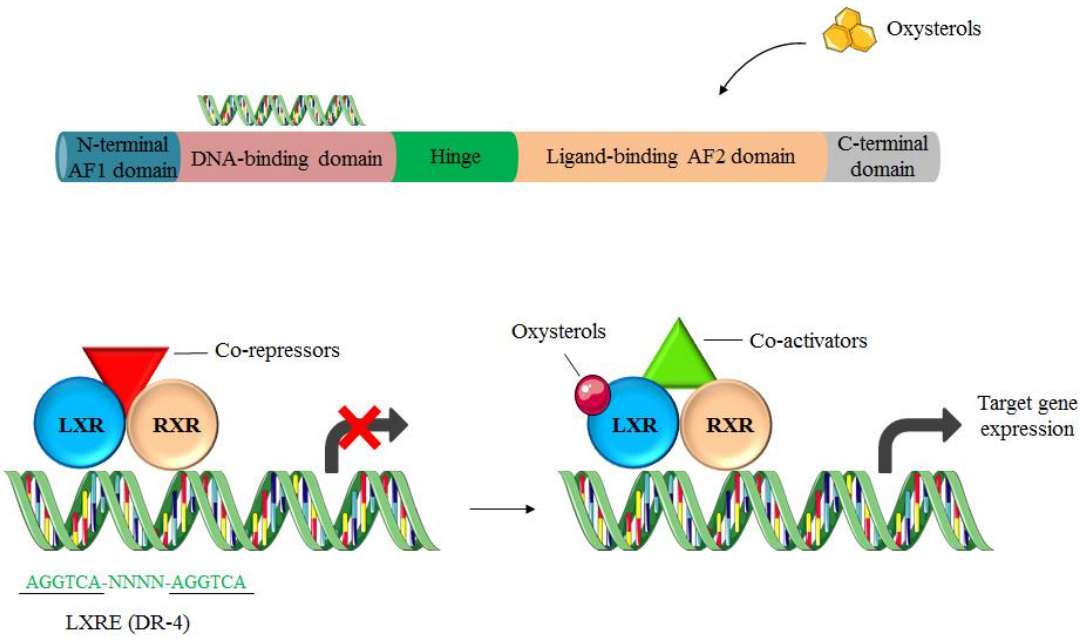


FIGURE 1.2: Modular structure and mechanism of action of LXR. Liver X receptor (LXR) has a conserved modular structure, comprising a DNA-binding domain and a ligand-binding domain joined by a hinge region. The activation function domain 1 (AF1) is located at the amino terminus (N-terminus) whereas the activation function domain 2 (AF2) is part of the ligand-binding domain. LXR forms an obligate heterodimer with retinoid X receptor (RXR) that binds to a direct repeats (DR4) LXR response elements (LXRE) in the regulatory regions of target genes, thereby repressing gene expression. Following ligand binding to LXR or RXR, the heterodimer changes its conformation and this leads to the release of co-repressors and the recruitment of co-activators that drive transcription.

repeats of the same consensus sequence without any spacer region (IR-0) or separated by 1 nucleotide (IR-1).

Similar to other members of the nuclear receptor superfamily which heterodimerise with RXR, LXRs reside within the nucleus and in the absence of a ligand remain pre-bound to cognate LXREs and in complex with corepressors such as the nuclear receptor co-repressor (N-CoR) (Chen and Evans, 1995) and the silencing mediator for retinoic acid and thyroid hormone receptor (SMRT) (Horlein et al., 1995) which block transcription by recruiting of histone deacetylases. Upon ligand-induced activation, LXR undergoes a conformational change that potentiates the release of the co-repressors and at the same time reinforces interactions with co-activators (e.g. steroid receptor coactivator 1 (SRC-1) (Son and Lee, 2010), peroxisome-proliferator-activated receptor gamma co-activator 1 α/β (PGC1α/β) (Oberkofler et al., 2003, Laffitte et al., 2003a) and transformation/transcription domain-associated protein (TRRAP) (Unno et al., 2005)) thereby inducing the transcription of target genes .

1.4.6 LXR and reverse cholesterol transport

LXR regulates whole-body cholesterol homeostasis by potently driving reverse cholesterol transport (Figure 1.3). *In vivo* studies have demonstrated that pharmacological activation of LXR in mice increases the circulating levels of HDL and promotes net cholesterol excretion (Repa et al., 2000). Studies in lipid-loaded macrophages have shown that LXR stimulates the expression of ABCA1 and ABCG1 in order to reduce the intracellular cholesterol burden. ABCA1 is a transmembrane protein which mediates the removal of cholesterol and phospholipids to lipid-poor plasma carriers such as ApoA1 (Repa et al., 2000). The importance of ABCA1 for cholesterol homeostasis is underscored by the fact that mutation of this gene in humans causes Tangier disease which is a condition characterised by excessive cholesterol deposition in peripheral tissues and negligible concentrations of plasma HDL (Singaraja et al., 2003). LXR agonists robustly stimulate the expression of ABCA1 due to the presence of an LXRE in the proximal promoter of this gene. Moreover, the increase in ABCA1 expression in response to cholesterol overload is strictly LXR-dependent as evidenced by the loss of this effect in in macrophages isolated from LXR-deficient mice (Repa et al., 2000). Furthermore, despite the fact that the liver is the major source of plasma HDL, intestinal enterocytes also play a key role in the biogenesis of this lipoprotein. *In vivo* studies have demonstrated that 30% of circulating HDL is derived from the intestine in an ABCA1-dependent manner (Brunham et al., 2006a). This pathway does not play a role in the primary and secondary absorption of the luminal cholesterol and instead it protects the body from sterol overaccumulation (it prevents cholesterol packaging into chylomicrons and its subsequent distribution into peripheral tissues by effluxing it into HDL and targeting it for biliary clearance) (Brunham et al., 2006a). LXR

directly controls this pathway and therefore administration of an intestine-specific LXR agonist to mice increases intestinal ABCA1 gene expression by six fold and plasma HDL-cholesterol levels by almost 50% (Brunham et al., 2006b) .

ABCG1 is a LXR target gene which works cooperatively with ABCA1 to promote cholesterol efflux from peripheral tissues into HDL particles. *In vitro* assays in cultured macrophages have demonstrated that activation of LXR increases the expression of this protein and thereby facilitates the transfer of cholesterol in to HDL_2 and HDL_3 particles (Wang et al., 2004). ABCG1 was initially postulated to be localised at the plasma membrane (Vaughan and Oram, 2005). However, recent studies have shown that it functions primarily as an intracellular transporter that facilitates the trafficking of cholesterol to the plasma membrane at the expense of esterification (Tarling and Edwards, 2011). A recent study has expanded this idea by suggesting that ABCG1 mediates the formation of extracellular cholesterol-rich domains; this type of lipid exportation not only protects the cell from sterol-induced cytotoxicity but also makes free cholesterol available for mobilisation by both ApoA1 and HDL (Freeman et al., 2014).

Adenosine diphosphate-ribosylation factor-like 7 (ARL7) is another gene whose promoter sequence comprises an LXRE and as such is a direct target of LXR. *In vitro* studies in cultured macrophages have shown that ARL7 transports cholesterol to the plasma membrane for ABCA1-associated removal and thereby plays an integral role in the LXR-dependent efflux pathway (Hong et al., 2011).

Moreover, LXR drives reverse cholesterol transport also by inducing a subset of lipoprotein particles which serve as plasma cholesterol acceptors; LXR drives the expression of the entire ApoE/C1/C2/C4 gene cluster in macrophages (Mak et al., 2002) and ApoD in adipose tissue (Hummasti et al., 2004). In more detail, LXR binds to conserved response elements in the multienhancer regions of the ApoE gene and directly regulates its expression (Laffitte et al., 2001). It is recognised that ApoE plays a key role in sterol homeostasis: ApoE-deficient mice develop severe hypercholesterolemia, primarily due to elevated levels of VLDL and IDL, and early-onset atherosclerosis (Plump et al., 1992). LXR is indispensable for the induction of ApoE in macrophages and adipose tissue in response to free cholesterol overload (Laffitte et al., 2001) and thereby plays a central role in sterol clearance in peripheral tissues.

In addition, LXR mediates cholesterol efflux by determining the activity of apolipoprotein- and lipid-modulating enzymes which are also essential for reverse cholesterol transport. Specifically, it has been demonstrated that the genes encoding phospholipid transfer protein (PLTP) (Laffitte et al., 2003b), LPL (Zhang et al., 2001) and human CETP (Luo and Tall, 2000)

harbour LXREs and as such are bona fide targets of the nuclear receptor. The coordinated regulation of these genes by LXR is believed to facilitate efficient free cholesterol clearance from macrophages, however the exact mechanism remains elusive.

1.4.7 LXR and cholesterol uptake

LXR controls cholesterol homeostasis by regulating the uptake of this sterol into the liver and extrahepatic tissues (Figure 1.3).

SR-B1 is a membrane-bound glycoprotein, most highly expressed in liver, which mediates the selective and efficient uptake of cholesteryl esters specifically from HDL, without the internalisation and degradation of the particle itself (Acton et al., 1996). *In vivo* studies have reported that overexpression of SR-B1 in the liver decreases plasma HDL and ApoA1 levels, stimulates the hepatic uptake of HDL-cholesterol and also increases biliary cholesterol levels (Kozarsky et al., 1997); the opposite phenotype is observed in mice lacking the scavenger receptor in their livers (Varban et al., 1998). *In vitro* assays in polarised hepatocyte system have also suggested that in cholesterol-depleted cells, SR-B1 is primarily localised on the basolateral surface, where it can access circulating lipoproteins, however, in response to cholesterol loading, it undergoes a slow transcytosis to the apical bile canaliculus where it facilitates the secretion of cholesterol into bile (Harder et al., 2007). The promoter region of SR-B1 harbours a conserved LXRE and *in vitro* studies have demonstrated that pharmacological activation of LXR increases the protein expression of this scavenger receptor in human hepatocytes (Malerod et al., 2002). Consequently, LXR plays a central role in the regulation of reverse cholesterol transport not only by driving the efflux of the sterol from peripheral tissues but also by directly promoting its hepatic clearance.

LDLR is a transmembrane glycoprotein which, after recognising ApoB-100, mediates the binding and endocytosis of cholesterol-rich LDL particles (Brown and Goldstein, 1986). Subsequently, LDL is trafficked to the lysosomal compartment where it is degraded while the released cholesteryl esters are hydrolysed to free cholesterol; free cholesterol could then be incorporated into the plasma membrane of the cells, re-esterified for intracellular storage or removed for biliary clearance (Brown and Goldstein, 1986). The LDLR, on the other hand, is either metabolised or recycled back to the plasma membrane (Brown and Goldstein, 1986). LXR, activated in response to intracellular overload with free cholesterol, is able to limit further uptake of the sterol by upregulating the expression of the inducible degrader of LDLR (IDOL) (Zelcer et al., 2009). IDOL is an E3 ubiquitin ligase which binds directly to the cytoplasmic tale of LDLR and promotes its polyubiquitination by the UBE2D1/E1 complex, thereby targeting the receptor for lysosomal degradation (Calkin et al., 2011, Zhang et al., 2011). Although LDLR

is expressed in a multitude of cell types, the ability of LXR to regulate it varies in a tissue- and species-specific manner. Specifically, previous studies have convincingly demonstrated that overexpression of IDOL in mouse liver reduces the protein levels of LDLR and raises the plasma cholesterol levels (Zelcer et al., 2009), whereas targeted deletion of IDOL in cultured mouse cells leads to increased LDLR protein levels and enhanced LDL uptake (Scotti et al., 2011). However, *in vivo* studies in mice have shown that pharmacological activation of LXR promotes IDOL expression only in extrahepatic tissues (e.g. macrophages) and not in the liver (Hong et al., 2014). In agreement with this, it has been observed that IDOL-deficient mice have increased LDLR expression only in extrahepatic tissues (Hong et al., 2014). In contrast, pharmacological activation of LXR in monkeys induces hepatic IDOL expression, reduces LDLR protein levels, and raises plasma LDL levels; knockdown of IDOL in primates blunts the effect of the LXR stimulation on LDL levels (Hong et al., 2014). These data strongly suggest that LXR could play even more central role in the control of cholesterol homeostasis in humans than it does in mice by limiting LDL-cholesterol uptake and thus protecting both the liver and extrahepatic tissues from cytotoxicity.

1.4.8 LXR and biliary cholesterol excretion

LXR function is of major importance for the control of whole-body cholesterol homeostasis due to the fact that it controls the sole routes for terminal elimination of the sterol into feaces, namely biliary excretion and transintestinal cholesterol efflux (Figure 1.3).

LXR modulates the biliary removal of neutral sterols by inducing the expression of ATP-binding cassette (ABC) transporters G5 (ABCG5) and G8 (ABCG8) (Repa et al., 2002). ABCG5 and ABCG8 are half-transporters, localised at the canalicular membrane of hepatocytes, which form an obligate functional heterodimer that mediates the efflux of hepatic free cholesterol into bile (Berge et al., 2000). Disruption of either ABCG5 or ABCG8 or both in mice results in diminished biliary cholesterol concentrations and significantly increased hepatic cholesterol accrual upon sterol challenge (Yu et al., 2002b, Plosch et al., 2004, Wang et al., 2007b). Mutations in these genes in humans cause sitosterolemia, an autosomal recessive disorder characterised by hypercholesterolemia and impaired biliary excretion of dietary sterols (Berge et al., 2000). Conversely, mice overexpressing ABCG5 and ABCG8 present with five times higher biliary cholesterol concentrations than wild-type controls and a compensatory increase in *de novo* cholesterol biosynthesis (Yu et al., 2002c). LXR directly regulates the expression of ABCG5/ABCG8 by binding to conserved response elements in the promoter sequences of these genes (Repa et al., 2002). Mice challenged with cholesterol-rich diet or with synthetic LXR agonists present with increased hepatic mRNA levels of these transporters whereas this effect is lost in the absence of LXR (Repa et al., 2002).

In mice, LXR promotes biliary cholesterol clearance by directly inducing CYP7A1 which catalyses the rate limiting step of the conversion of cholesterol into bile acids (Peet et al., 1998). LXRα-deficient mice are unable to induce the expression of CYP7A1 and therefore present with hepatic accumulation of cholesteryl esters as a consequence of their diminished ability to metabolise cholesterol to bile acids (Peet et al., 1998). Conversely, LXRβ is also expressed in the liver but mice lacking this receptor do not display an obvious hepatic phenotype even when challenged with a high-cholesterol diet (Alberti et al., 2001), indicating that LXRα is the dominant isoform in this tissue. Interestingly, the LXRE found in the promoter sequence of rodent CYP7A1 is not conserved in humans (Chiang et al., 2001), reinforcing the idea the LXR orchestrates cholesterol homeostasis in a species-dependent manner.

As previously mentioned (refer to 1.4.5) LXR also promotes biliary cholesterol excretion by inducing the expression of the scavenger receptor SR-B1.

Moreover, LXR stimulates transintestinal cholesterol efflux. *In vivo* studies have demonstrated that in wild-type mice the excretion of blood-derived free cholesterol directly into the intestinal lumen represents 33% of the total neutral sterol faecal loss, whereas pharmacological activation of LXR strongly stimulates this pathway (raising its contribution to 63%) possibly by modulating the expression of the ABCG5 and ABCG8 transporters which play a critical role in this process (van der Veen et al., 2009). These observation have also been confirmed in mouse models which are unable to secrete cholesterol into bile (Kruit et al., 2005).

1.4.9 LXR and intestinal cholesterol absorption

LXR coordinates cholesterol homeostasis also by controlling the absorption of cholesterol from the diet (Figure 1.3). LXR modulates this pathway by indirectly regulating the expression of Niemann-Pick C1-Like 1 (NPC1L1) (Duval et al., 2006). NPC1L1 is a polytopic transmembrane protein which in mice primarily enriched in the small intestine and is in the brush border membrane of enterocytes (Altmann et al., 2004). Five out of its thirteen transmembrane domains constitute a sterol-sensing domain which is necessary to bind cholesterol (Ohgami et al., 2004). *In vitro* studies have demonstrated that NPC1L1 protein cycles between intracellular compartments and the cell membrane in a cholesterol-dependent manner: when the cells are enriched with cholesterol, NPC1L1 resides predominantly in the endocytic recycling compartment whereas when the cells are sterol-depleted, it moves to the apical plasma membrane in order to shuttle cholesterol from the intestinal lumen into the enterocytes (Yu et al., 2006). Mice deficient in NPC1L1 exhibit diminished cholesterol absorption (Altmann et al., 2004). Interestingly, in humans NPC1L1 is also expressed in the liver, where it specifically co-localises with ABCG5/ABCG8 at the canalicular membrane of

hepatocytes (Temel et al., 2007). Studies in mice overexpressing human NPC1L1 protein in their livers have demonstrated that biliary cholesterol excretion is dramatically reduced in these animals, thereby suggesting that hepatic NPC1L1 prevents excessive loss of cholesterol into bile (Temel et al., 2007). Both *in vitro* and *in vivo* studies have demonstrated that pharmacological activation of LXR reduces the expression of NPC1L1 (Duval et al., 2006) thereby reducing primary cholesterol absorption under conditions of cholesterol excess.

Moreover, LXR limits cholesterol absorption in the intestine by regulating the expression of ABCG5 and ABCG8 transporters at the intestinal brush border membrane (Repa et al., 2002). In addition to their role in biliary cholesterol excretion (reviewed in 1.4.6), these proteins mediate the apical efflux of cholesterol out of the enterocytes and into the intestinal lumen, resulting in increased faecal sterol excretion (Berge et al., 2000). Mutations in ABCG5 and ABCG8 in humans cause sitosterolemia which is associated with heightened absorption of sterols and hypercholesterolemia (Berge et al., 2000). However, disruption of these half-transporter complexes in mice does not raise primary absorption of dietary cholesterol and plasma cholesterol levels, instead these rodents present with enhanced fractional absorption of plant sterols and dramatically elevated plasma and tissue levels of plant sterols (Yu et al., 2002b). Conversely, overexpression of human ABCG5 and ABCG8 in mice causes 50% reduction in fractional absorption of dietary cholesterol and up to six fold increase in faecal neutral sterol excretion (Yu et al., 2002c). Mice challenged with cholesterol-rich diet or with synthetic LXR agonists present with raised clearance of cholesterol into faeces as a consequence of the enhanced expression of ABCG5 and ABCG8 in the intestine; this effect is lost in the absence of LXR (Repa et al., 2002).

Furthermore, LXR controls intestinal absorption of dietary cholesterol by managing the composition of the bile acid pool. It has been established that the degree of hydrophobicity of the bile acid pool is directly proportional to cholesterol uptake from diet as a consequence to the fact that bile acids higher hydrophobicity (e.g. CA) are more effective in intraluminal micellar cholesterol solubilisation (Wang et al., 2003). Natural muricholic acids (MCAs) are highly hydrophilic and as such are the most powerful inhibitors of cholesterol absorption (Wang et al., 2003). Over 95% of the murine biliary bile acid pool is composed of cholic and muricholic acids, and their ratio is tightly controlled so that upon administration of high-cholesterol feeding, the MCA proportion increases in order limit excessive uptake of the sterol (Peet et al., 1998). However, this response to cholesterol challenge is completely lost in LXRα-deficient mice (Peet et al., 1998). Also, the substantial increase in the CA/MCA ratio seen in these mice suggests that these animals may absorb more dietary cholesterol as result of the defective LXR-signalling (Peet et al., 1998).

1.4.10 LXR and cholesterol biosynthesis

LXR regulates cholesterol homeostasis and protects the cells from over-accumulation of this sterol by reducing *de novo* cholesterol biosynthesis (Figure 1.3). Specifically, *in vitro* studies have demonstrated that LXR binds directly to negative response elements located in the genes encoding lanosterol 14α-demethylase (CYP51A1) and squalene synthase (farnesyl diphosphate farnesyl transferase 1(FDFT1)), both of which are key cholesterologenic enzymes that belong to the mevalonate pathway; oxysterol-mediated activation of LXR decreases the mRNA expression of these genes (Wang et al., 2008).

1.4.11 LXR and de novo lipogenesis

Availability of free fatty acids is crucial for cholesterol homeostasis due to the fact that they are used as substrates for esterification of the sterol. Cholesterol esterification protect mammalian cells from cytotoxicity since it limits the solubility of the sterol in membrane lipids and promotes the sequestration of sterol in cytoplasmic lipid droplets (Nelson and Cox, 2013b). LXR protects the cells from overaccumulation of free cholesterol by inducing *de novo* lipogenesis and thereby enhancing the formation of cholesteryl esters (Figure 1.3). Specifically, LXR stimulates lipogenesis indirectly by inducing the transcription factor sterol regulatory element-binding transcription factor 1c (SREBP1c) which then drives the expression of a plethora of enzymes involved in fatty acid biosynthesis (Schultz et al., 2000). *In vivo* studies have also shown that pharmacological activation of LXR increases triglyceride levels in the liver and plasma and this effect is lost in mice deficient in this nuclear receptor (Schultz et al., 2000). Moreover, LXR enhances lipogenesis in a SREBP1c-independent manner by directly binding to response elements in the promoter sequences of key enzymes mediating fatty acid biosynthesis (fatty acid synthase (FAS) (Joseph et al., 2002), stearoyl-coenzyme A desaturase 1 (SCD1) (Chu et al., 2006) and acetyl-coenzyme A carboxylase 1 (ACC1) (Talukdar and Hillgartner, 2006)), thereby stimulating their expression and activity.

In conclusion, LXR plays an encompassing role in the regulation of cholesterol homeostasis, namely control of *de novo* biosynthesis, primary and secondary absorption, influx and efflux, esterification and clearance. However, there is a significant gap in our understanding of how LXR functions in the physiological settings of pregnancy and whether the activity of this nuclear receptor is altered in order to facilitate the development of gestational hypercholesterolemia. Part of the novel work described in this document will aim to expand the knowledge on this topic.

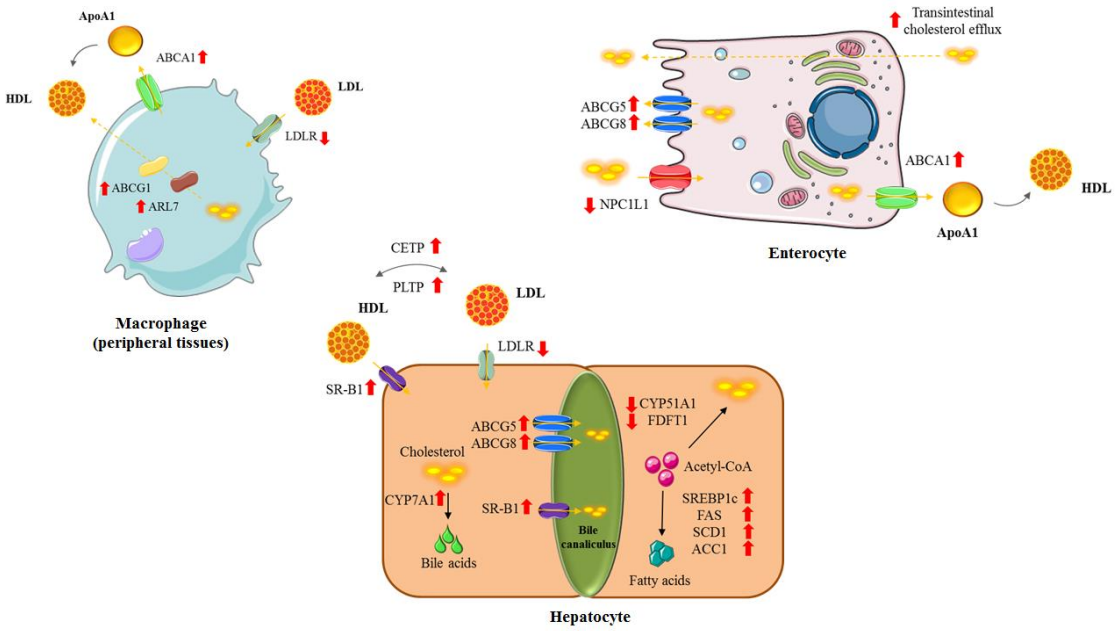


FIGURE 1.3: Tissue-specific role of LXR in the control of cholesterol homeostasis.

LXR protects the cells from free cholesterol-induced cytotoxicity by controlling cholesterol homeostasis in a tissue-specific manner. In peripheral tissues, such as macrophages, LXR promotes cholesterol efflux by upregulating the expression of ABCA1 (adenosine triphosphate-binding cassette A1), ABCG1 (adenosine triphosphate-binding cassette G1) and ARL7 (adenosine diphosphate-ribosylation factor like 7) which facilitate the HDL-dependent transfer of the sterol to the liver. LXR reduces the utilisation of LDL-cholesterol into these cells by downregulating the expression of LDLR (LDL receptor). Activation of LXR in the intestine limits the absorption of dietary and biliary cholesterol by decreasing the expression NPC1L1 (Niemann-Pick C1-Like 1) and upregulating the transporters ABCG5/8 (adenosine triphosphate-binding cassette G5/8) at the apical membranes of enterocytes. In the same tissue, LXR promotes the efflux of cholesterol from cells and its transfer to the liver via HDL particles. Activation of LXR in the intestine also facilitates the utilisation of circulating cholesterol across the basal enetrocytic membranes and its apical efflux into the intestinal lumen. In the liver, LXR promotes uptake of HDL- and LDL-cholesterol via SR-B1 (scavenger receptor B1) and LDLR respectively. LXR also stimulates the clearance of cholesterol from hepatocytes and into bile canaliculi via the ABCG5/8 and SR-B1 transporters. In mice, LXR promotes cholesterol conversion to bile acids by CYP7A1 (cytochrome P450 7A1). LXR facilitates cholesterol esterification and storage by inducing the expression of the fatty acid biosynthetic genes SREBP1c (sterol regulatory element-binding transcription factor 1c), FAS (fatty acid synthase), SCD1 (stearoyl-coenzyme A desaturase 1), ACC1 (acetyl-coenzyme A carboxylase 1). In the liver, LXR reduces *de novo* cholesterol biosynthesis by downregulating the expression of

CYP51A1 (lanosterol 14α-demethylase) and FDFT1 (farnesyl diphosphate farnesyl transferase 1). LXR also modulates the cholesterol content of lipoprotein particles by upregulating the expression of CETP (cholesteryl ester transfer protein) and PLTP (phospholipid transfer protein).

1.5 Adipose tissue

Adipose tissue is a complex organ with profound impact on metabolism and energy homeostasis. It is mainly composed of mature adipocytes and stromal vascular cells, including preadipocytes, fibroblasts, vascular endothelial cells and a variety of immune cells (e.g. macrophages). In mammals, there are two major types of adipose tissue, white and brown.

1.5.1 Brown adipose tissue

Brown adipose tissue (BAT) is a highly vascularised and mitochondria-rich organ whose main function is thermogenesis via the uncoupling of mitochondrial respiration from adenine triphosphate (ATP) production. In humans, BAT appears early in embryological development, becomes morphologically and biochemically identifiable at birth and starts to regress following infancy. It has been theorised that human babies have significant brown fat depots, presumably to provide heat in the cold environment encountered at birth (Cannon and Nedergaard, 2004). Although it has been considered that adult humans are devoid of BAT unless specifically challenged by chronic cold or by cancer-related states of catecholaminergic excess, positron emission tomography using radiotracers such as [18F]-2-fluoro-D-2-deoxy-D-glucose has revealed that adults have active brown depots in the neck and chest region, as well as in the kidney area (Nedergaard et al., 2010). Further studies have shown that 30-100% of adults have detectable BAT, with no difference between the genders (Nedergaard et al., 2010). In rodents, brown adipocyte accumulations are located in the intrascapular and perirenal regions (Cannon and Nedergaard, 2004).

Morphologically, brown adipocytes have polygonal or ellipsoid shapes and their maximum diameter varies between 15µm-50µm (Cinti, 2012). These cells are multiocular, i.e. they store triglycerides in the form multiple small lipid droplets, and contain numerous big mitochondria, packed with transverse cristae. Also, BAT is highly vascularised and this is due in part to the increased demand for oxygen in response to the enhanced metabolic activity of the tissue. It has been suggested that the increased capillary density facilitates rapid heat dissipation and prevents any heat-dependent cellular damage. BAT is also well innervated, with numerous noradrenergic fibres stemming from the sympathetic nervous system. Brown adipocytes are highly specialised cells that dissipate stored chemical energy in the form of heat, a process termed adaptive thermogenesis (Enerback et al., 1997). When thermogenesis is needed, the sympathetic nervous system releases norepinephrine which activates beta-adrenergic receptors at the plasma membrane of brown adipocytes thereby initiating a protein kinase A (PKA) signalling cascade which culminates in the conversion of triglycerides into free fatty acids that then induce the activity of uncoupling protein 1 (UCP1) (Nicholls and Locke, 1984). UCP1 is an integral

protein localised in the inner mitochondrial membrane which catalyses a proton leak across this lipid bilayer thereby "uncoupling" fuel oxidation from ATP synthesis; it has been proposed that UCP1 acts as a long-chain fatty acid/H^+ symporter (Fedorenko et al., 2012).

Although BAT plays a key role in the metabolism of rodents, its contribution to the physiology of adult humans remains debatable.

1.5.2 White adipose tissue biology

White adipose tissue (WAT) is an organ highly adapted to storing surplus fatty acids derived from the diet in the form of triglycerides and subsequently releasing them under conditions of negative energy balance in the body. WAT has important cushioning function, serving to protect joints exposed to high mechanical stress (palms and soles) and to shield delicate organs (retro-orbital fat) (Rosen and Spiegelman, 2014). Also, it plays an important role in streamlining aquatic mammals and in providing insulation (Rosen and Spiegelman, 2014).

Morphologically, white adipocytes are spherical uniocular cells which comprise a single cytoplasmic lipid droplet that occupies approximately 90% of total cellular volume. The rest of the cytoplasm contains the nucleus, which is "squeezed" by the central vacuole and stays flattened at the periphery of the cell, under-developed Golgi apparatus, ER, few lysosomes, and few elongated mitochondria with short, randomly arranged cristae (Cinti, 2011).

In humans, WAT starts developing in the beginning of the second trimester of pregnancy and expands rapidly after birth as a result of both increased adipocyte number (hyperplasia) and adipocyte size (hyperotrophy) (Poissonnet et al., 1983). The ability of preadipocytes to proliferate and differentiate decreases with age and therefore it is believed that increased fat cell volume as a consequence of lipid storage is the main route for WAT expansion in adult humans (Bjorntorp, 1974). However, recent studies have demonstrated that 10% of fat cells are renewed annually suggesting that there is continuous adipogenesis and constant turnover of fat cells in adulthood (Spalding et al., 2008). Even though human adipocytes conglomerate differently in distinct parts of the body, forming depots with specific morphological and functional properties, the most common classification schemes distinguish between subcutaneous (under the skin) and visceral (inside the body cavity) fat, and this an oversimplification stemming from the fact that the latter is strongly associated with metabolic syndrome. In rodents, WAT develops mainly after birth and is organised into two subcutaneous depots and several visceral depots. Interestingly, many depots in humans have no precise correlates in rodents and vice versa. For example, epididymal WAT is a large depot in male mice, routinely studied as a representative of visceral fat, which is absent in men (Rosen and Spiegelman, 2014). Moreover, in humans the

majority of visceral fat is contained in the omentum, which is barely present in rodents (Rosen and Spiegelman, 2014).

Nevertheless, studies in both humans and mice have convincingly demonstrated that there are pronounced molecular, anatomical and overall physiological differences between subcutaneous and visceral fat (described in detail in section 1.5.9), and therefore these two depots have unique contributions energy and lipid metabolism.

1.5.3 Overview of fatty acid metabolism

Fatty acids are the major building block of all fats and oils that function as major energy depositories in animals. They are carboxylic acids with hydrocarbon chains varying between 2 and 36 carbon atoms. In some fatty acids the chain is unbranched and contains no double bonds (polysaturated fatty acids) whereas in others the chain comprises one or more double bonds (polyunsaturated fatty acids). Fatty acid composition is species- and tissue-specific. In animal and plant tissues, fatty acids with 16 and 18 carbon atoms are most abundant (e.g. palmitic, stearic, oleic and linoleic acids) while the ones with chain lengths shorter than 14 and longer than 22 carbon atoms are present only in minor concentrations (Nelson and Cox, 2013c, Tvrzicka et al., 2011).

Fatty acids have a variety of essential physiological functions. They are a principal source of energy and play a pivotal role in energy metabolism. Specifically, triglycerides are non-polar hydrophobic molecules which are composed of three fatty acids each in an ester linkage to a single glycerol backbone. Triglycerides are more efficient as energy fuels due to the fact that they are unhydrated and their oxidation yields more than twice as much energy, gram for gram, as the oxidation of carbohydrates due to the fact that the carbon atoms of fatty acids are more reduced than those of sugars. Moreover, fatty acids are precursors for a plethora of molecules and thereby play key roles in the structure and function of cell membranes (e.g. phospholipids and sphingolipids) and intracellular signalling cascades (e.g. phosphatidylinositol and phosphatidylcholine). Finally, they are substrates for the formation of hormones and signalling molecules that can influence signal transduction pathways and gene transcription (Nelson and Cox, 2013c, Tvrzicka et al., 2011).

In humans, fatty acids could be obtained exogenously from the diet and endogenously from hepatic biosynthesis and adipocyte release. In eukaryotic cells, fatty acid biosynthesis is a four-step process, taking place in the cytosol, which mediates the conversion of acetyl-CoA and malonyl-CoA into palmitate by the multienzyme complex fatty acid synthase; acetyl-CoA carboxylase catalyses the rate-limiting step of this pathway, namely the formation of malonyl-

CoA from acetyl-CoA. Palmitate is the precursor for other long-chain fatty acids which are generated following further additions of acetyl groups by fatty acid elongation systems present in the ER and mitochondria (Nelson and Cox, 2013b). In contrast to the liver, human WAT has a low potential for *de novo* lipogenesis (Shrago et al., 1969), except under conditions of overfeeding with high-carbohydrate diet (Hellerstein et al., 1996), therefore it is recognised that fatty acid biosynthesis in WAT depots has negligible contribution to the overall energy balance. In rodents, however, WAT *de novo* lipogenesis is higher than in humans and also the expression of lipogenic enzymes such ACC1 and FAS is under nutritional and hormonal regulation (Girard et al., 1994).

Exogenous fatty acids are supplied to the body as dietary triglycerides. Similar to cholesterol (refer to section 1.4.2), ingested triglycerides are emulsified by the action of the amphipathic bile salts that act as biological detergents, converting dietary fats into mixed micelles of bile salts and triglycerides. Micelle formation increases the fraction of lipid molecules accessible to the action of water-soluble lipases in the intestine. Lipase action converts triacylglycerols to monoacylglycerols and diacylglycerols, free fatty acids and glycerol which are then transferred across the luminal membrane of the intestinal epithelial cells. Inside the enterocytes, dietary lipids get reesterified into triglycerides and packaged into chylomicrons. As previously described, peripheral tissues, such as WAT and skeletal muscle, take up free fatty acids following LPL-mediated hydrolysis of triglycerides circulating as components of chylomicron, VLDL, IDL and LDL particles (Figure 1.1.)(Nelson and Cox, 2013a).

Finally, fatty acids are catabolised to produce chemical energy via mitochondrial β-oxidation. Fatty acids which have fourteen or more carbons enter the mitochondria with the help of a carnitine shuttle. Specifically, fatty acids get activated to fatty acyl-CoA by the action of acyl-CoA synthetase and then get trans-esterified to carnitine at the outer mitochondrial membrane or in the intermembrane space via the action of carnitine acyltransferase 1. The product of these reactions, fatty acyl-carnitine, then enters the matrix by facilitated diffusion through the acyl-carnitine/carnitine transporter of the inner mitochondrial membrane. In the matrix, carnitine dissociates from the fatty acyl-CoA and is transported back to the intermembrane space. Carnitine acyltransferase 1 catalyses the rate-limiting step of the β-oxidative pathway. The activity of this enzyme is inhibited by malonyl-CoA, ensuring that fatty acid biosynthesis and degradation will not happen simultaneously, which otherwise would have caused futile waste of energy. Subsequently, acetyl-CoA groups, produced by β-oxidation, enter the Krebs cycle where they get oxidised to produce carbon dioxide and release ATP. Although mitochondrial β-oxidation is by far the most important catabolic fate for fatty acids in animal cells, there is another pathway in some species, including vertebrates, called ω-oxidation. The enzymes unique to ω-oxidation are located in the endoplasmic reticulum of liver and kidney and

preferentially target fatty acids with 10 or 12 carbon atoms. In mammals, ω-oxidation is normally a minor pathway for fatty acid degradation but when β-oxidation is defective it becomes essential. Also, there are branched-chain fatty acids which could not undergo β-oxidation (e.g. phytanic acid) and therefore are catabolised in the peroxisomes of animal cells via a pathway called α-oxidation. The importance of this pathway is revealed in patients suffering from Refsum's disease who have impaired α-oxidation and therefore present with neurologic damage, cerebellar degeneration, and peripheral neuropathy as a result of phytanic acid tissue accumulation (Nelson and Cox, 2013a).

1.5.4 WAT metabolism: fatty acid uptake and trafficking

When the body is at rest and under eucaloric conditions, WAT is the main tissue which takes up fatty acids and glycerol removed from circulating lipoproteins. Due to their hydrophobic nature, fatty acids that have been released into the blood bind non-covalently to albumin which acts as a soluble protein carrier; each albumin monomer could carry up to ten fatty acids. In WAT, fatty acids dissociate from albumin, diffuse across the outer aqueous phase and then insert into the outer leaflet of the adipocyte membrane. The exact mechanism of how fatty acids manage to cross the lipid bilayer is a subject of a debate. A theory for a diffusion-mediated transfer (flip-flop) of fatty acids suggests that these molecule undergo an extremely rapid passive transport across the lipid bilayer followed by a slower dissociation from the membrane and into the cytosol. This concept is based on studies that demonstrate rapid fatty acid flip-flop rates in small unilamellar vesicles. The opposing theory, which is more widely accepted, proposes that lipid uptake is predominately protein-mediated. Several transmembrane proteins have been implicated in the transmembrane traffic of fatty acids including FABPpm, FAT/CD36, FATP1 and FATP4, Acyl CoA Synthetase Long Chain 1, and caveolin 1 (Thompson et al., 2010). Finally, fatty acids that leave the cytosolic leaflet of the plasma membrane are then bound and sequestered by fatty acid binding proteins (e.g. activating protein 2 (aP2)) and acyl-CoA binding proteins which facilitate their intracellular trafficking (Matarese et al., 1989).

In addition to fatty acids, WAT is also able to utilise and store glucose. In particular, adipocytes express the insulin-dependent glucose transporter 4 (GLUT4), a peptide which mediates the postprandial flux of glucose from the circulation into the cell thus supplying glycolytic intermediates, such as glycerol-3-phosphate and acetyl-CoA, to the triglyceride biosynthetic pathway (Mueckler and Thorens, 2013).

1.5.5 WAT metabolism: TG biosynthesis and storage

In adipocytes, fatty acids taken up from the circulation are converted into triglycerides via a multistep mechanism and are then packaged into cytoplasmic lipid droplets, which consist of neutral lipid core enveloped by a polar phospholipid monolayer associated with lipid droplet-specific proteins (Figure 1.4). The hydrophobic nature of triglycerides strongly facilitates their storage by allowing them to aggregate into tight and compact clusters which are unsolvated and also do not raise the osmolarity of the cytosol. Due to their relative chemical inertness, these lipid molecules can be stored in large quantities in cells without the risk of undesired chemical reactions with other cellular constituents (Nelson and Cox, 2013b).

The first commitment step of triglyceride synthesis is the esterification of fatty acyl-CoA with glycerol-3-phosphate to form 1-acylglycerol-3-phosphate (lysophosphatidic acid), a reaction catalysed by glycerol-3-phosphate acyltransferase. The vast majority of the glycerol 3-phosphate is derived from the glycolytic intermediate dihydroxyacetone phosphate by the action of the cytosolic glycerol-3-phosphate dehydrogenase. On the other hand, acyl-CoA is derived from fatty acids that are activated by acyl-CoA synthetase. Next, lysophosphatidic acid is further esterified and converted into phosphatidic acid in the reaction catalysed by 1-acylglycerol-3-phosphate acyltransferase. Phosphatidic acid is hydrolysed by phosphatidic acid phosphatase to form a 1, 2-diacylglycerol which is then then converted to triacylglyceride by transesterification with a third fatty acyl–CoA (Nelson and Cox, 2013b).

All the enzymes involved in triglyceride biosynthesis are localised in the ER. However, the exact mechanisms mediating the packaging of lipids into droplets and promoting the dissociation of these structures from the ER are still elusive. The most widely accepted model of lipid droplet formation is that lipids accumulate between the cytosolic and the luminal leaflets of the phospholipid bilayer of the ER, followed by association of lipid droplet-specific proteins and eventual separation of the growing cluster from the ER.

Moreover, it has been demonstrated that the proteins coating the intracellular lipid droplets contribute to whole-body energy metabolism by actively regulating the turnover of triglycerides in white adipocytes. The PAT family, including perilipin, adipose differentiation-related protein (ADRP) and tail-interacting protein 47 (TIP47) and S3-12, are the best characterised lipid droplet-associated proteins which share sequence homology but nevertheless differ in terms of tissue distribution, subcellular localisation and lipid binding properties. Perilipin and ADRP are constitutively associated with mature lipid droplets and in the absence of neutral lipids they get targeted for proteosomal degradation. In contrast, TIP47 and S3-12 are stable cytosolic proteins when the intracellular lipid pool is quiescent while, upon active triglyceride biosynthesis they

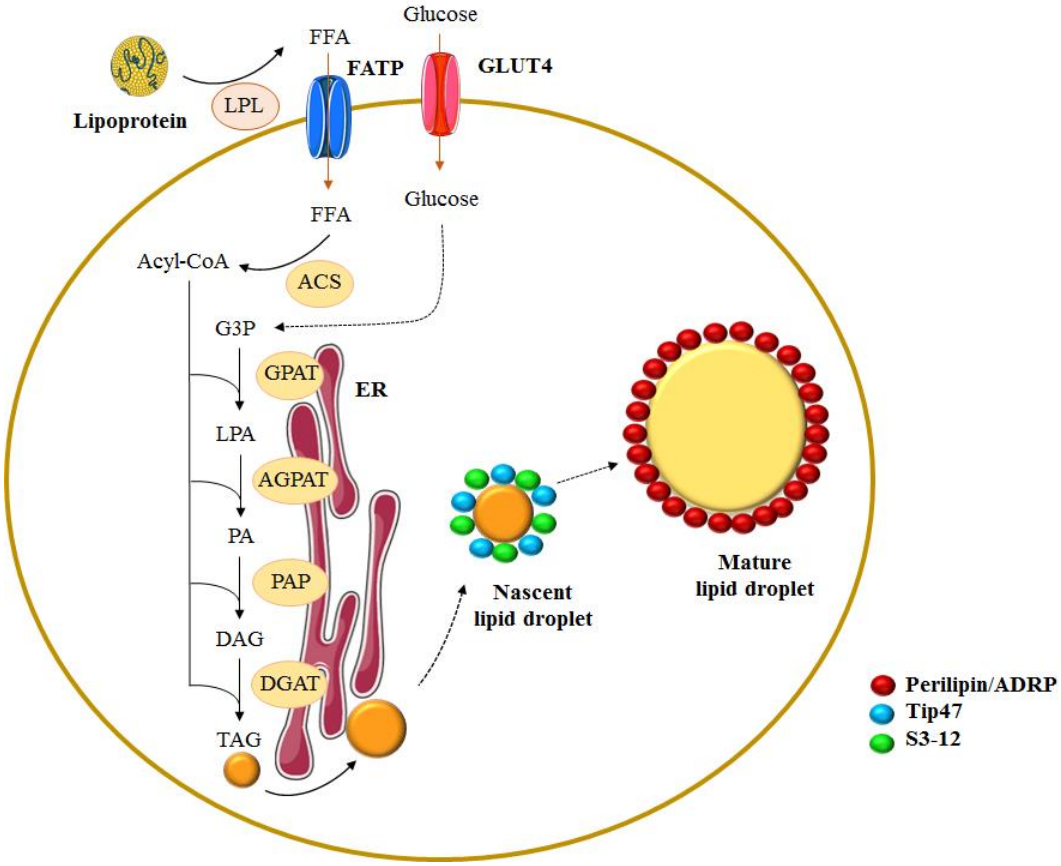


FIGURE 1.4: Adipose tissue lipogenic pathway. FFA, free fatty acids; LPL, lipoprotein lipase; FATP, fatty acid transfer protein; GLUT4, glucose transporter 4; ACS, acyl-CoA synthetase ; G3P, glycerol 3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, 1, 2-diacylglycerol; TAG, triacylglycerol; GPAT, glycerol-3-phosphate acyltransferase; AGPAT, 1-acylglycerol-3-phosphate acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, diacylglycerol acyltransferase; ADRP, adipose differentiation-related protein; Tip47, tail-interacting protein 47; ER, endoplasmic reticulum.

translocate and coat the newly-formed lipid droplets that emerge from the endoplasmic reticulum. It has been proposed that TIP47 and S3-12 mediate the delivery of nascent lipid droplets to the perilipin/ADRP-delineated storage depots (Bickel et al., 2009). Studies have demonstrated that perilipin is the principal regulator of triglyceride homeostasis in adipocytes and its genetic ablation results in reduced adiposity as well as diminished hormone-regulated lipolysis (Tansey et al., 2001). Furthermore, it has been shown that perilipin functions as an activator of fat-specific protein 27 (Sun et al., 2013), a member of the CIDE family which is pivotal for the directional transfer of lipids from small to large uniocular lipid droplets (Nishino et al., 2008).

1.5.6 WAT metabolism: TG mobilisation

WAT is a lipid pool which is in a constant state of flux resulting from largely futile cycles of lipolysis and re-esterification that allow for its fine tuning of this organ in response to metabolic demands (Kalderon et al., 2000). Under instances of energy deprivation, there is a shift towards a greater net rate of lipolysis which results in the liberation of three non-esterified fatty acids and a glycerol moiety into the circulation to be utilised by other organs (e.g. skeletal muscle, heart and renal cortex) as energy substrates (Figure 1.5). Triglyceride breakdown is catalysed in stepwise manner by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL). According to the most recent model of hormone-stimulated lipolysis, signalling via β-adrenergic receptors stimulates adenylyl cyclase, thus increasing the levels of cyclic adenosine monophosphate (cAMP) and activating PKA. PKA then phosphorylates perilipin which causes the recruitment of CGI-58 and subsequent activation of the lipid droplet-bound ATGL. Moreover, following PKA-mediated phosphorylation, HSL translocates from the cytoplasm to the lipid droplet where together with ATGL they hydrolyse triacylglycerols to diacylglycerols and monoacylglycerols. Monoacylglycerols are subsequently converted to free fatty acids and glycerol by MGL (Thompson et al., 2010).

Studies in both humans and rodents have demonstrated that catecholamines (epinephrine and norepinephrine) are the major activator of lipolysis and bind to the β-adrenergic receptors located at the plasma membrane of adipocytes (Galton and Bray, 1967). Glucagon, a pancreatic hormone which signals low glucose levels, is also able to stimulate fat hydrolysis (Perea et al., 1995). Moreover, it has been reported that glucocorticoids (Xu et al., 2009), thyroid hormone (Elks and Manganiello, 1985), sex steroids (testosterone in males (Xu et al., 1991)) and cytokines (e.g. TNF-α) (Ryden et al., 2002)) are able to enhance lipolysis through various mechanisms. In contrast, insulin modulates WAT lipolysis by suppressing it. Secreted by pancreatic beta-cells in response to elevated glucose levels, insulin stimulates signalling

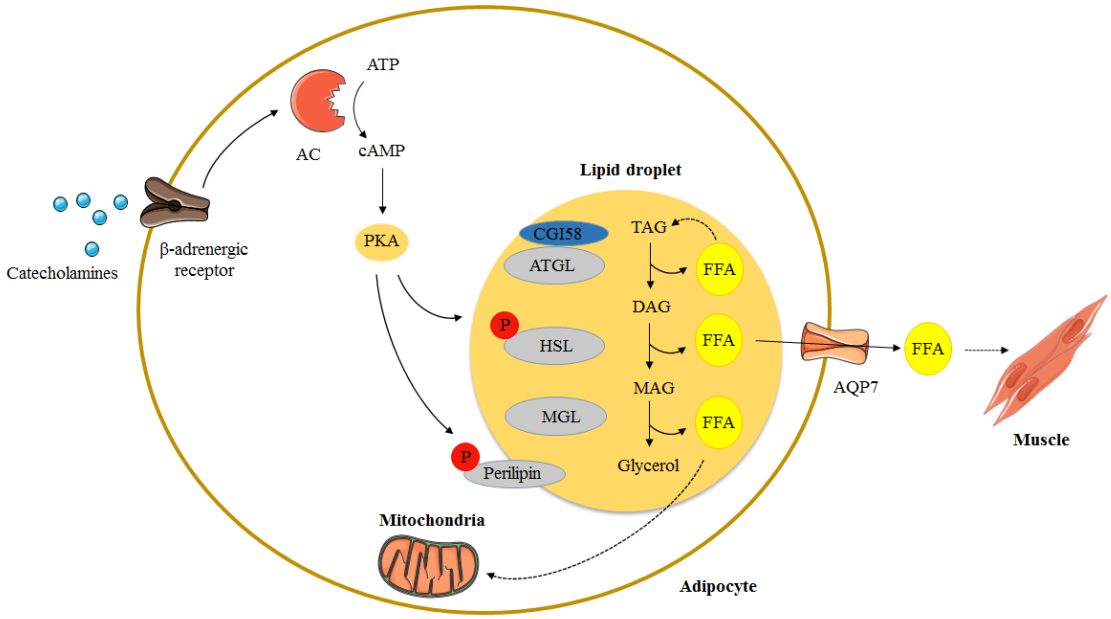


FIGURE 1.5: Adipose tissue lipolytic pathway. FFA, free fatty acids; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CGI58, comparative gene identification 58; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; MGL, monoacylglycerol lipase; TAG, triacylglycerol; DAG, diacylglycerol; MAG, monoacylglycerol; AQP7, aquaporin 7.

pathways which result in the degradation of adipocyte cAMP by phosphodiesterase 3B and also cause protein phosphatase-1-mediated dephosphorylation of HSL (Stralfors and Honnor, 1989). In addition, insulin is shown to downregulate ATGL and promote fatty acid re-esterification (Kershaw et al., 2006).

1.5.7 WAT as endocrine organ

WAT functions not only as a storage depot but also as an endocrine organ. The discovery of leptin in 1994 (Zhang et al., 1994) revolutionises the idea of white fat as a passive depot which stores excess energy and instead today WAT is recognised as a dynamic organ that communicates with other tissues by receiving signals and secreting a variety of hormones and metabolites, termed adipokines, which regulate whole-body lipid and glucose homeostasis.

Leptin is an adipocyte-secreted peptide which regulates energy homeostasis by signalling to the brain and peripheral tissues primarily via the janus-kinase/signal transducer and activator of transcription-3 pathway (Tartaglia et al., 1995). Leptin is a "satiety hormone" which directly activates or inhibits neurons in the hypothalamic area of the brain that controls hunger and thereby promotes reduction in food intake (Cowley et al., 2001, Spanswick et al., 1997). Also, there is a positive linear correlation between circulating levels of serum leptin and total body fat mass, which can be explained by increased release of leptin from large compared to small fat cells. Defects in leptin signalling and expression are associated with the development of hyperphagia, hypothermia, insulin resistance, reproductive and immune dysfunction as well as early onset morbid obesity in both humans (Ozata et al., 1999) and mice (Zhang et al., 1994). These data demonstrate the potent effect of this adipokine not only on the brain but also on various peripheral tissues which express the leptin receptor. *In vivo* studies have demonstrated that in addition to its effect on hunger, leptin also stimulates 5′-AMP-activated protein kinase (AMPK) signalling in skeletal muscle and thereby not only promotes fatty oxidation and glucose uptake but also prevents lipid accumulation in this tissue (Minokoshi et al., 2002). It has been determined that early activation of the AMPK pathway occurs by leptin acting directly on muscle, whereas later activation depends on leptin functioning through the hypothalamic-sympathetic nervous system axis (Minokoshi et al., 2002). Furthermore, studies in rodents have shown that leptin is able to reduce the biosynthesis of monounsaturated fatty acids in the liver by specifically repressing the expression and activity of SCD1 (Cohen et al., 2002).

Adiponectin is an abundant plasma protein that is also secreted exclusively from the adipose tissue. With exception of severe cases of undernutrition (e.g. women suffering from anorexia nervosa (Iwahashi et al., 2003)) and newborn infants (Lindsay et al., 2003)), there is a strong inverse correlation between plasma adiponectin concentration in humans and fat mass (Hu et al.,

1996), with obesity reducing adiponectin levels while weight reduction increases adiponectin (Ouchi et al., 1999, Matsubara et al., 2002). Adiponectin has been shown to improve whole-body insulin sensitivity in models of genetic and diet-induced obesity (Yamauchi et al., 2001). Specifically, *in vitro* studies have demonstrated that this adipokine increases insulin sensitivity by inducing AMPK signalling which reduces mTOR/S6 kinase activity and limits the inhibitory phosphorylation of insulin receptor substrate 1 (Wang et al., 2007a). Also, adiponectin stimulates fatty acid oxidation and glucose uptake in skeletal muscle and adipose tissue in an AMPK-dependent manner (Yamauchi et al., 2002). Another important role of this peptide is the suppression of hepatic glucose output. *In vivo* studies have shown that adiponectin activates an AMPK signalling cascade in hepatocytes and consequently inhibits both the expression of gluconeogenic enzymes and the rate of endogenous glucose synthesis in these cells (Combs et al., 2001). Adiponectin has no effect on the rates of glucose uptake, glycolysis, or glycogen synthesis in the liver (Combs et al., 2001). Moreover, this adipokine has been shown to stimulate the appetite and reduce energy expenditure by promoting AMPK-signalling in the hypothalamus in rodents (Kubota et al., 2007). Also, the observation that leptin sensitivity is markedly increased in adiponectin-deficient mice suggests that leptin and adiponectin function reciprocally to control energy homeostasis (Kubota et al., 2007).

Resistin is another WAT-derived secretory factor which plays a significant role in obesity-induced insulin resistance. It is released by adipocytes in rodents (Steppan et al., 2001) and macrophages in humans (Patel et al., 2003) and its synthesis is raised after feeding and under conditions of obesity. Infusion or over-expression of resistin leads to hyperglycemia which is largely due to enhanced hepatic gluconeogenesis (Banerjee et al., 2004, Qi et al., 2006). Conversely, reducing plasma resistin levels by deleting the resistin gene, infusing resistin antibodies or resistin antisense oligodeoxynucleotides all protect against obesity-induced hyperglycemia primarily by restoring hepatic insulin responsiveness (Steppan et al., 2001, Banerjee et al., 2004). However, the importance of resistin in humans is debatable as a consequence of the fact that increases in the circulating levels of this adipokine have not been consistently reported in obese patients with type 2 diabetes (Sentinelli et al., 2002). On the other hand, mutations in the resistin promoter region which lead to elevated resistin gene transcription are also associated with increased levels of serum resistin, obesity and insulin resistance (Osawa et al., 2004).

Retinol binding protein-4 (RBP4) is an adipokine which plays a key role in the regulation of insulin sensitivity. Specifically, *in vivo* studies have shown that RBP4 expression is increased in the adipose tissue of mice which lack GLUT4 in this tissue and are therefore insulin resistant (Yang et al., 2005). Conversely, the levels of this adipokine are reduced in insulin-sensitive mice which overexpress GLUT4 in fat (Yang et al., 2005). Moreover, injection of recombinant

human RBP4 into lean mice as well as overexpression of RBP4 in transgenic rodents cause insulin resistance and glucose intolerance whereas RBP-deficient mice appear to be protected from developing high fat diet-induced insulin resistance (Yang et al., 2005). RBP4 has been shown to cause systemic insulin resistance by impairing insulin signalling in muscle (Yang et al., 2005) and adipocytes (Ost et al., 2007), and also promoting hepatic gluconeogenesis (Yang et al., 2005). Furthermore, several studies in both humans and rodents have demonstrated that there is strong direct correlation between raised RBP4 serum levels and obesity (Graham et al., 2006, Yang et al., 2005); it has been proposed that RBP4 contributes to many aspects of metabolic syndrome including inflammation, fatty liver disease and insulin resistance.

As previously mentioned, WAT comprises many non-adipocyte cells including immune cells that are able to secrete various cytokines, which not only affect adipose tissue function in a paracrine manner but also signal to other peripheral tissues such as skeletal muscle and liver and thereby control whole body energy homeostasis. It has been postulated that lean adipose tissue is predominantly infiltrated by alternatively activated M2 macrophages which secrete anti-inflammatory signals such as araginase and interleukin 10, and promote tissue repair and angiogenesis (Lumeng et al., 2007). Expansion of adipose tissue leads to adipocyte hypertrophy and the release of chemokines (e.g. monocyte chemoattractant protein 1 and colony-stimulating factor 1) that induce that recruitment of classically activated M1 macrophages which secrete proinflammatory cytokines (Lumeng et al., 2007). Proinflammatory cytokines alter the gene expression profiles of adipocytes and impair their insulin sensitivity (Lumeng et al., 2007). These changes in WAT contribute to systemic insulin resistance by changing the patterns of adipokine secretion and increasing lipolysis which raises the levels of circulating NEFAs.

TNFα is a classic example of adipose tissue-secreted cytokine which plays a key role in the regulation of lipid metabolism. Synthesised as a 26 kDa transmembrane protein that undergoes cleavage by a metalloproteinase, it is released into the circulation as a 17 kDa soluble TNFα molecule (Kriegler et al., 1988). Since mature adipocytes are capable of producing TNFα, it has been proposed that they are the principal source of elevated TNFα levels in obesity. However, more recently it has been recognised that macrophages from the stromal vascular fraction are the primary source of adipose derived TNFα and that the increased levels of this cytokine in obesity are due to the increased infiltration of adipose tissue with M1 macrophages (Weisberg et al., 2003). TNFα is the first adipose-derived factor proposed to be the link between obesity, inflammation and diabetes. Specifically, the mRNA expression of TNFα is increased in obese adipose tissue and this cytokine has since been strongly implicated in the pathogenesis of insulin resistance. A number of studies have demonstrated that TNFα can impair insulin signalling in hepatocytes and adipose tissue (Stephens et al., 1997, Ruan et al., 2002, Cai et al., 2005) and also impair insulin-stimulated glucose uptake in rat skeletal muscle. The molecular

basis for the observed impairment in insulin action involves inhibition of IRS signalling capability through the activation of serine kinases such as the c-Jun-N-terminal kinase or inhibitor of nuclear factor kappa-light-chain-enhancer of activated B cells kinase (IKK) and through increased expression of suppressor of cytokine signalling 3 (Steinberg et al., 2006a). Moreover, it has been shown that targeted deletion of TNFα or its receptors increased insulin sensitivity and glucose tolerance in obese rodents (Uysal et al., 1997). TNFα neutralization does not appear to improve glucose tolerance or insulin sensitivity in obese type 2 diabetic humans, however prolonged treatment with this cytokine does improve insulin sensitivity in obese individuals without established type 2 diabetes (Tam et al., 2007). Furthermore, TNFα reduces fatty acid oxidation in hepatocytes (Nachiappan et al., 1994) and skeletal muscle (Steinberg et al., 2006b) through effects mediated by the induction of protein phosphatase 2C and suppression of AMPK (Steinberg et al., 2006b). The reduced rates of fatty acid oxidation are accompanied by increased accumulation of bioactive lipids which in turn are known to activate protein kinase C and inhibit insulin receptor substrate function (Yu et al., 2002a).

In addition to the adipokines and cytokines mentioned in this section, WAT produces a broad spectrum of peptides which affect the metabolism and function of different organs and tissues including muscle, liver, vasculature and brain. Endocrine signals produced by WAT play essential roles in the modulation of whole-body energy and nutrient homeostasis and therefore are substantially implicated not only in the development of metabolic dysfunction in the context of disease (such as obesity and type 2 diabetes) but also in the establishment of "metabolic dysfunction-like phenotype" observed in physiological conditions such as pregnancy.

1.5.8 Overview of the role of peroxisome proliferator-activated receptor γ in the control of WAT metabolism

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the nuclear receptor superfamily of ligand-activated transcription factors and shares the same modular structure (refer to section 1.4.4) (Mangelsdorf et al., 1995). It associates with RXR forming a permissive heterodimers which bind to DNA response elements called PPAR response elements (PPREs), the consensus being repeats of the sequence AGGTCA separated by one or two nucleotides (DR-1 or DR-2 respectively) (Kliewer et al., 1992). PPARγ is most highly expressed in WAT and BAT, where it is a master regulator of adipogenesis as well as a potent modulator of whole-body lipid metabolism and insulin sensitivity (Tontonoz and Spiegelman, 2008). Due to alternative splicing and differential promoter usage, PPARγ exists as two isoforms, PPARγ1 and PPARγ2, with the latter containing an additional 30 amino acids at its N-terminus (Tontonoz and Spiegelman, 2008). Whereas PPARγ1 is expressed in many tissues, the

expression of PPARγ2 is restricted to adipose tissue under physiological conditions but can be induced in other tissues by a high-fat diet (Medina-Gomez et al., 2007).

Even though polyunsaturated fatty acids (e.g. linoleic and arachidonic acids) and their derivatives (e.g. prostanoids and oxidised fatty acids) have been identified as endogenous ligands of PPARγ, these molecules have very low affinity for the receptor and are able to activate it only when administered in supraphysiological concentrations (Tontonoz and Spiegelman, 2008). In contrast, synthetic ligands, such as thiazolidinediones (TZDs) are potent activators of PPARγ and are used clinically as anti-diabetic agents enhancing insulin sensitivity (Kung and Henry, 2012).

PPARγ is a master regulator of adipogenesis and its expression increases during the differentiation of preadipocytes into adipocytes (Tontonoz et al., 1994a). Gain–of–function experiments have demonstrated that ectopic expression of this nuclear receptor alone is sufficient to initiate the entire adipogenic program in non-adipogenic mouse fibroblasts, including expression of adipose-specific genes followed by morphological differentiation (Tontonoz et al., 1994b). Moreover, both *in vivo* and *in vitro* experiments have shown that PPARγ is physiologically indispensable (Rosen et al., 2002). Since homozygous PPARγ-deficient mice are not viable due to the role that PPARγ plays in trophoblast formation (Kubota et al., 1999), tetraploid rescue of PPARγ-null mutants and chimeric mice made from PPARγ$^{-/-}$and PPARγ$^{+/+}$ blastocysts have been used to show that no adipocytes develop from PPARγ-null cells and as a consequence these animals are completely devoid of adipose tissue (Kubota et al., 1999, Barak et al., 1999). Furthermore, the effects of PPARγ mutations in humans also underscore the important role of this transcription factor in human adipose tissue development and function. Specifically, heterozygous mutations in the ligand-binding domain of this nuclear receptor cause severe insulin resistance as well as early-onset type 2 diabetes and hypertension (Barroso et al., 1999). On the other hand, in humans, one gain-of-function mutation of PPARγ has been identified and all of the affected patients present with severe obesity, supporting the idea of positive correlation between PPARγ activity and adiposity (Hamer et al., 2002). PPARγ is also essential for mature adipocyte function, as revealed by the finding that adipocytes only survive for a few days after selective ablation of the nuclear receptor (Imai et al., 2004).

In mature adipocytes, PPARγ plays a pivotal role in the control of fatty acid and glucose uptake, lipogenesis and insulin sensitivity. Specifically, it binds to response elements located in upstream enhancer regions of genes controlling these pathways such as GLUT4, LPL, aP2 and phosphoenolpyruvate carboxykinase (PEPCK) (Tontonoz et al., 1994a, Tontonoz et al., 1995). Moreover, PPARγ controls the expression of numerous adipokines and cytokines secreted from

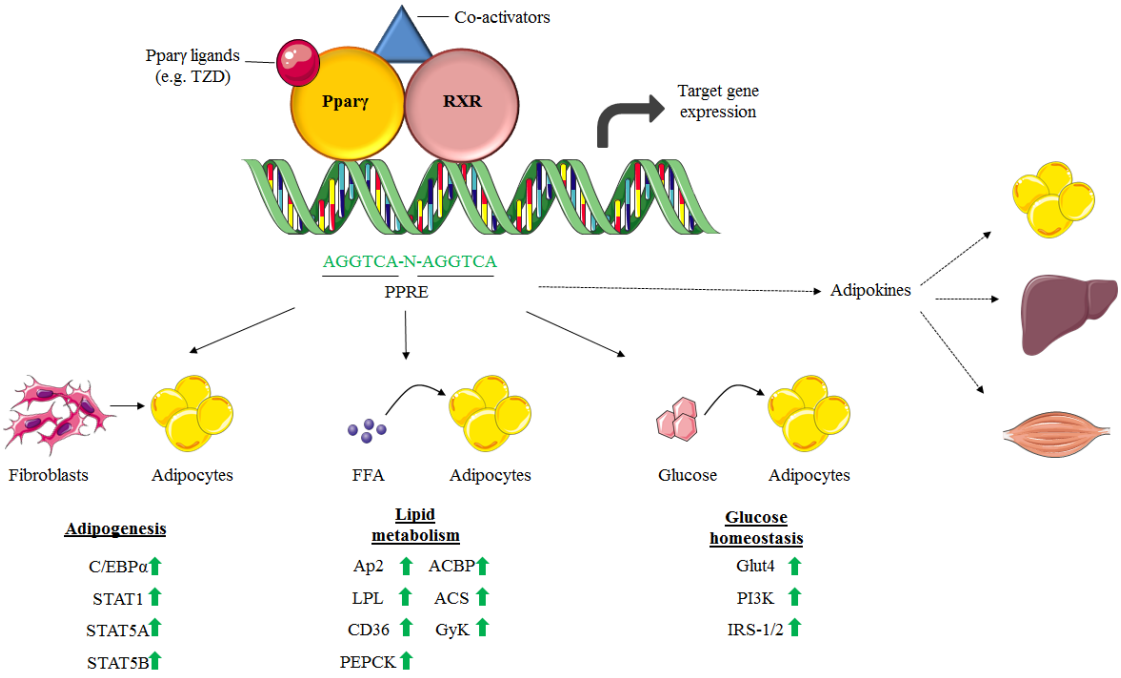


FIGURE 1.6: Role of PPARγ in the regulation of adipocyte metabolism. Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear receptor which forms and obligate heterodimer with the retinoid X receptor (RXR). Upon ligand activation (e.g. thiazolidinediones (TZDs)), the PPARγ-RXR heterodimer maintains adipose tissue homeostasis by binding to PPAR response elements (PPREs) and directly regulating the expression of genes involved in adipogenesis, lipid metabolism and glucose homeostasis. PPARγ promotes the secretion of adipokines which act as signal transducers that affect the physiological activities of peripheral organs such as liver and muscle as well as of adipose tissue itself. C/EBPα, CCAAT/enhancer-binding protein α; STAT 1/ 5A/ 5B, signal transducers and activators of transcription 1, 5A, 5B; FFA, free fatty acids; Ap2, activating protein 2; ACBP, acyl-CoA-binding protein; LPL, lipoprotein lipase; CD36, cluster of differentiation 36; PEPCK, phosphoenolpyruvate carboxykinase; ACS, acetyl-CoA synthetase; GyK, glycerol kinase; GLUT4, glucose transporters 4; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; IRS-1/2, insulin receptor substrate 1/2.

adipose tissue, such as adiponectin, resistin, leptin and TNFα which also influence insulin sensitivity (Figure 1.6) (Ahmadian et al., 2013).

1.5.9 Subcutaneous versus visceral adipose tissue

Unlike other organs, white adipose tissue appears in multiple discrete locations throughout the body. It has been conclusively demonstrated that WAT distribution is of crucial clinical importance since visceral fat accumulation is associated with elevated risk of insulin resistance, type 2 diabetes, dyslipidaemia, hypertension, atherosclerosis, hepatic steatosis and overall mortality. Consistent with the notion that visceral WAT has adverse implications on metabolic health, omentectomy (removal of visceral fat) results in decreased insulin and glucose levels in humans (Thorne et al., 2002). By contrast, peripheral obesity (fat accumulation predominantly in the subcutaneous compartment) is associated with improved insulin sensitivity and a lower risk of developing type 2 diabetes (Snijder et al., 2003). *In vivo* studies in rodents have confirmed that transplanting subcutaneous fat into the visceral compartment results in decreased serum cholesterol and triglyceride levels, improved hepatic and peripheral insulin sensitivity, and increased life span in animal models whereas placement of visceral fat into subcutaneous compartment has no beneficial effect (Tran and Kahn, 2010). However, the exact molecular mechanisms underlying the regulation of regional adiposity and the disproportionate effects of enlargements in visceral fat depots on human health remain enigmatic. Nevertheless, many studies have described depot differences in physiology, cellular composition and endocrine functions which could help explain the differential contributions of subcutaneous and visceral WAT to the control of lipid metabolism and energy homeostasis.

To begin with, subcutaneous and visceral WAT differ from an anatomical perspective. Specifically, subcutaneous WAT accumulates 80% of total body fat and is highly specialised in long-term energy storage since it offers unrestricted peripheral expansion (Ibrahim, 2010, Wajchenberg, 2000). In contrast, visceral fat accounts for 10-20% of fat in men and 5-8% in women, and provides short-term energy storage that can be rapidly accessed by the liver; visceral WAT has very limited potential for expansion due to the physical constraints imposed on it by the abdominal cavity and the internal organs (Wajchenberg, 2000, Ibrahim, 2010). It has been theorised that the greater accrual of subcutaneous fat in females has evolved to facilitate steady access to calories during pregnancy whereas large visceral fat stores have developed in males to allow for swift access to energy during hunting. Gender-specific patterns in fat distribution could also be explained by differences in steroid hormone receptor densities between the two depots. Namely, adipocytes isolated from visceral WAT present with higher androgen receptor density whereas subcutaneous WAT adipocytes are more densely populated with estrogen receptors (Pedersen et al., 1996, Ibrahim, 2010). Therefore, hormonal

manipulations to convert males to females cause redistribution of fat from visceral to subcutaneous depot, while the opposite occurs in female-to-male transsexuals (Thomou et al., 2010). Nevertheless, the effects of sex steroids on fat distribution are not straightforward as evidenced by the fact that women with polycystic ovarian syndrome are hyperandrogenic and present with visceral obesity while testosterone administration to hypogonadal males reverses visceral obesity (Lee et al., 2013).

Moreover, regional differences in WAT metabolism could be partially attributed to the unique innervation and relationship with the blood circulation. For example, the venous drainage of visceral fat enters the portal circulation thereby bathing the liver with free fatty acids and adipokines while the venous drainage of subcutaneous fat goes into the systemic circulation. Also, visceral WAT is reported to be more vascularised and more heavily innervated than subcutaneous WAT (Ibrahim, 2010).

Furthermore, differences in the functionality of fat depots could be explained by differences in their cellularity. Previous studies have established that small adipocytes are more insulin-responsive and have a higher propensity for fatty acid uptake thereby preventing ectopic lipid deposition whereas large adipocytes are insulin-resistant and hyperlipolytic. Once fat cells become too large, they become necrotic, stimulating macrophage infiltration. Differences in cell size between the fat depots mainly arise from differences in their mechanisms for tissue expansion. Namely, subcutaneous WAT responds to energy overload by increasing fat cell number and therefore this depot is populated with small and thermodynamically stable adipocytes which avidly respond to hormonal signals. In contrast, visceral fat enlarges through hypertrophy, consistent with its role in storing and releasing nutrients rapidly and its limited space for growth (Ibrahim, 2010, Thomou et al., 2010).

Regional differences in fatty acid uptake and storage could determine the depot-specific roles of white fat to lipid metabolism. *In vivo* experiments have shown that the direct uptake of labelled free fatty acids is greater in subcutaneous WAT as compared to visceral fat (Marin et al., 1996). It has also been reported that visceral adipocytes have a higher propensity for glucose uptake than the subcutaneous ones (Ibrahim, 2010).

Also, fat depots could have differential contributions to lipid homeostasis due to their unique lipolytic potentials. Visceral WAT presents with higher β_3-adrenergic receptor density and is more responsive to hormone-stimulated lipolysis than subcutaneous WAT; these two depots do not exhibit any differences in their rates of basal lipolysis. Visceral adipocytes are also less sensitive to α_2-adrenergic receptor- and insulin-dependent inhibition of lipolysis (Arner, 1995, Zierath et al., 1998). *In vitro* studies have suggested that the decreased response to insulin in

these cells could be due to the reduced protein expression of the insulin receptor substrate-1 (Zierath et al., 1998). Interestingly, subcutaneous and visceral adipocytes do not present with differences in insulin receptor binding capacity, mRNA and protein expression (Zierath et al., 1998).

Adipose tissue depots have a unique impact on whole-body energy homeostasis as a consequence to their distinct glycoprotein, adipokine and paracrine factor secretion profiles. Specifically, it has been demonstrated that the mRNA levels and protein secretion of leptin is higher in subcutaneous than visceral WAT, and the circulating levels of this adipokine are more closely correlated to subcutaneous fat mass (Montague et al., 1997, Van Harmelen et al., 1998). Subcutaneous adipocytes produce more RBP-4 (Kos et al., 2011) whereas visceral WAT secretes more adiponectin (Motoshima et al., 2002). Also, macrophage infiltration is more extensive in visceral than subcutaneous fat and therefore this depot presents with higher expression of pro-inflammatory cytokine such as TNFα, C-reactive protein, interleukin-6 and monocyte chemotactic protein 1 (Fontana et al., 2007, Ibrahim, 2010).

Finally, regional variations in adipose tissue metabolism cannot be attributed solely to extrinsic influences (e.g. anatomical location, abundance of non-adipose cells and hormonal microenvironment) and instead they also result from differences in the inherent characteristics of preadipocyte populations. *In vitro* studies have shown that preadipocytes, isolated from different fat depots of the same individual and then cultured under identical conditions, are able to retain distinct depot-specific characteristics for weeks (Tchkonia et al., 2006). Such studies have demonstrated that subcutaneous preadipocytes have a replicative potential which is greater than that of visceral cells, is heritable (possibly via epigenetic modifications), does not depend on the presence of other cell types and does not relate to telomerase activity (Tchkonia et al., 2006). Moreover, subcutaneous preadipocytes possess a higher adipogenic capacity and are less susceptible to apoptosis than visceral cells (Tchkonia et al., 2002, Tchkonia et al., 2006). Genome-wide expression profiles in undifferentiated primary human preadipocytes, isolated from different WAT depots of the same donor, have shown that the unique dynamic properties of these cells result from the differential expression of more than 500 genes, 25% of which are involved in developmental processes (Tchkonia et al., 2007).

1.5.10 WAT adaptations in human pregnancy

As previously discussed (refer to sections 2.1.1 and 2.1.2), during human pregnancy the adaptations in WAT metabolism follow a biphasic pattern. The first two trimesters of gestation are centred on triglyceride deposition in maternal adipose tissues as a consequence of the enhanced expression and activity of endothelial LPL which hydrolyses circulating triglycerides

into free fatty acids making them available for adipocyte uptake (Alvarez et al., 1996). In late pregnancy, fat accrual declines, due to the decreased LPL activity in WAT (Alvarez et al., 1996) and stored lipids are mobilised, as a result of enhanced HSL expression (Martin-Hidalgo et al., 1994); these adaptations ensure continuous supply of glucose and lipids to support the development of the fetus and also provide the mother with sufficient energy substrates to meet the demands of pregnancy and prepare for lactation.

Moreover, a recent study aiming to describe the longitudal changes in subcutaneous WAT during non-complicated pregnancy, has demonstrated that gestational fat mass expansion results from both hyperplasia and hypertrophy (Resi et al., 2012). The same work also suggests that early-pregnancy lipogenesis results not only from the increased activity of endothelial LPL but also from the activation of PPARγ and Forkhead box protein O1 signalling pathways which promote enhanced adipocyte storage and increased preadipocyte differentiation. In addition, it has been demonstrated that there is raised expression of inflammatory markers accompanied by activation of the Toll-like receptor 4 signalling cascade in the adipose tissue of pregnant women suggesting that physiological inflammation is an early step which precedes the development of insulin resistance in adipocytes (Resi et al., 2012).

Furthermore, pregnancy affects the endocrine profile of WAT. Specifically, it has been reported that serum leptin concentrations increase significantly in early human gestation thereby preceding any major changes in maternal body fat composition and resting metabolic rate (Highman et al., 1998). Despite the fact that the circulating levels of this adipokine remain elevated until term, its ant-diabetic effects are absent in the mother. Therefore, it could be theorised that physiological pregnancy is associated with leptin resistance, a potentially crucial adaptation in the mother that allows for the maintenance of hyperphagia under conditions of concurrent fat accumulation. Also, circulating levels of adiponectin decrease throughout gestation, reaching a nadir during the third trimester, and thus strongly correlate with the loss of insulin sensitivity in the mother (Catalano et al., 2006). Hypoadiponectineamia is further exaggerated in pregnancies complicated with GDM, suggesting that this hormone could be a key factor signalling the adaptations in lipid metabolism throughout gestation. Moreover, studies in the lumbar adipose tissue (subcutaneous fat) of late pregnant rats have shown that there is increased expression of plasminogen activator inhibitor-1 (an acute phase protein that is elevated in the early stages of an inflammatory response) and TNFα expression in combination with activation of p38 mitogen-activated protein kinase signalling pathway and ERK phosphorylation cascade which contribute to the development of pregnancy-associated insulin resistance (reviewed also in 1.2.5) (de Castro et al., 2011).

Non-complicated human pregnancy is associated with increased circulating levels of pro-inflammatory cytokines (Curry et al., 2008, Hauguel-de Mouzon and Guerre-Millo, 2006). However, many of these molecules are secreted in large quantities from the placenta thereby masking the effect of pregnancy on the endocrine function of adipose tissue (Hauguel-de Mouzon and Guerre-Millo, 2006). Nevertheless, the excessive distribution of placenta-derived signalling peptides in maternal circulation does not preclude the possibility that WAT-secreted factors signal in an autocrine and paracrine manner to adipose tissues cells (both adipocytes and stromal vascular fraction components) thereby not only modulating local lipid metabolism but also regulating whole-body energy homeostasis.

In conclusion, WAT is a key metabolic tissue which regulates energy homeostasis not only by actively integrating lipid storage and lipid mobilisation pathways in response to whole-body energy demand but also by secreting hormones and signalling peptides that modulate the activity of other metabolic tissues such as skeletal muscle, liver and brain. Moreover, WAT is a non-homogeneous tissue since distinct fat depots have unique cellular compositions, adipogenic potential, lipid metabolism and endocrine profiles. Specifically, visceral and subcutaneous fat are shown to play contrasting roles in the control of lipid and carbohydrate metabolism under conditions of metabolic dysfunction. However, there is a substantial gap in our knowledge of whether visceral and subcutaneous WAT change differently in response to physiological pregnancy and if they could have unique contributions to the development of gestation-specific adaptations in lipid metabolism. Also, it is not known whether WAT dysfunction could play a role in the development of dyslipidaemia in pregnancies complicated with gestational cholestasis. Part of the novel work presented in this document will aim to expand the knowledge on these two topics.

1.6 Circadian rhythms and metabolism

In mammals, major components of energy homeostasis including the sleep-wake cycle, feeding, thermogenesis, glucose and lipid metabolism are subjected to circadian regulation which synchronises energy intake and expenditure in accordance with predictable daily changes in the environment.

1.6.1 Overview of circadian physiology

Adjustments in behavioural and physiological processes are orchestrated by the master pacemaker situated in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus which integrates environmental cues, called Zeitgebers, and then entrains the peripheral clocks present in metabolic organs (e.g. liver, WAT, skeletal muscle) through neuronal and hormonal signals.

In mammals, the daily light-dark cycle provides the principal entraining signal to the SCN which integrates photic information from the environment via neurons transcending from the retina via the retino-hypothalamic tract. The circadian system is organized hierarchically, meaning that while molecular oscillations occur in most cells and tissues of the body, the SCN functions as the master regulator, synchronizing the phase of other oscillating tissues to geophysical time. The role of the SCN as a master pacemaker is confirmed in rodents who permanently lose their diurnal patterns of behaviour and locomotion as a result of bilateral electrolytic lesions in the suprachiasmatic nuclei of their brains (Stephan and Zucker, 1972). Moreover, neural grafts from the suprachiasmatic region are able to restore circadian locomotor and feeding activities in arrhythmic animals whose own SCN had been ablated; the restored rhythms of the host always match the rhythms of the donor, demonstrating the strong impact of this nucleus on circadian activity (Ralph et al., 1990). On the other hand, peripheral clocks in metabolic tissues are also entrained by the central clock through feeding/ fasting cycles which alter the levels of key endocrine hormones and energy metabolites. *In vivo* studies have demonstrated that in mice restricted to a 4 hour feeding episode during their rest period (light phase) the diurnal metabolic activity of the liver becomes entrained to the new schedule of food availability within two days (Stokkan et al., 2001). Restricted feeding experiments have documented that although food-induced phase resetting proceeds faster in liver, other tissues such as lung, kidney, heart and pancreas are also able to entrain their metabolic activities in response to modified feeding/fasting patterns (Damiola et al., 2000); circadian activity of SCN is largely unaffected by changes in food availability and remains phase-locked to the light-dark cycle (Damiola et al., 2000, Stokkan et al., 2001). Moreover, in a liver which lacks normal circadian rhythmicity, the majority of hepatic gene expression rhythms can be restored by exposure to a temporally restricted feeding schedule (Vollmers et al., 2009). The restriction of feeding to a few hours during an animal's normal resting period results in food anticipatory activity, in which locomotion patterns deviate from normal circadian cycles. Such enhanced activity during the rest period serves as a preamble to the feeding event, indicative of the animal's anticipation of food consumption. Food anticipatory activity is a key adaptation which optimises the switch between daily anabolic and catabolic states in metabolic tissues aligning them in phase with periods of feeding and fasting. For instance, synchronisation between diurnal activities of the gastrointestinal tract and feeding patterns ensures that enzymes and transporters involved in nutrient absorption are expressed and available in anticipation of daily episodes of food ingestion (Panda et al., 2002).

1.6.2 Core clock machinery

Studies have shown that at the cellular level, circadian rhythmicity is controlled by several tissue-specific transcriptional-translational feedback loops (Figure 1.7). In particular, the basic

clock machinery consists of the transcriptional activators brain and muscle ARNTL-like protein 1 (BMAL1) and circadian locomoter output cycles kaput (CLOCK) which heterodimerise to positively regulate the expression of target genes Cryptochrome (CRY1 and CRY2) and Period (PER1, PER2, and PER3) and REV-ERBα. When the protein accumulation of PER and CRY reaches a certain threshold, they initiate a negative feedback loop by translocating to the nucleus as dimers and repressing the transcriptional activity of BMAL1/CLOCK. The nuclear receptors REV-ERBα/β form an accessory feedback loop by downregulating the expression of BMAL and functioning as key regulators of metabolic networks. The retinoid-related orphan receptor (ROR; RORα, RORβ, RORγ) are also considered part of the core clock machinery since they can drive the expression of BMAL and Rev-ERBα and are repressed by Rev-ERBα. Also, the intracellular levels of clock proteins are subject to posttranslational regulation by casein kinases (CKIε and CKIδ) and ubiquitin E3 ligases β-transducin repeat containing protein 1 (β-TrCP), F-box/LRR-repeat protein 3 (FBXL3), and adenosine diphosphate-ribosylation factor-binding protein 1 / protein associated with Myc (ARF-BP1/PAM) (Bass and Takahashi, 2010, Feng and Lazar, 2012, Eckel-Mahan and Sassone-Corsi, 2013).

To ensure a strict temporal regulation of various metabolic pathways, many key steps and players involved in these pathways are regulated by the circadian clock machinery. This regulation is achieved by controlling the expression of enzymes that regulate rate-limiting steps of a metabolic pathway, by integrating proteins such as nuclear receptors and nutrient-sensors with the clock machinery and by regulating the abundance of several metabolites. Additionally, the core clock machinery controls mammalian physiology and metabolism by employing translational and posttranslational regulation as well as remodelling of chromatin architechture which, however, are outside the scope of the work presented in this document and will not be discussed.

1.6.3 Circadian regulation of gene expression

Circadian clocks control physiology and nutrient homeostasis by directly regulating the expression of key metabolic genes. Transcriptomic studies in different mouse tissues have revealed that a large proportion of the whole transcriptomes (3-20%) undergo circadian oscillations. Moreover, circadian transcriptomes are tissue specific and tightly correlated with cellular functions. In the SCN they include genes involved in protein-neuropeptide synthesis, secretion, and degradation and regulation of locomotor activity whereas in the liver they comprise genes involved in glucose, lipid and xenobiotic metabolism (Panda et al., 2002).

Recent studies have demonstrated that the circadian clock modulates key metabolic pathways by regulating the expression of rate limiting enzymes. For example, HMGCR catalyses the rate

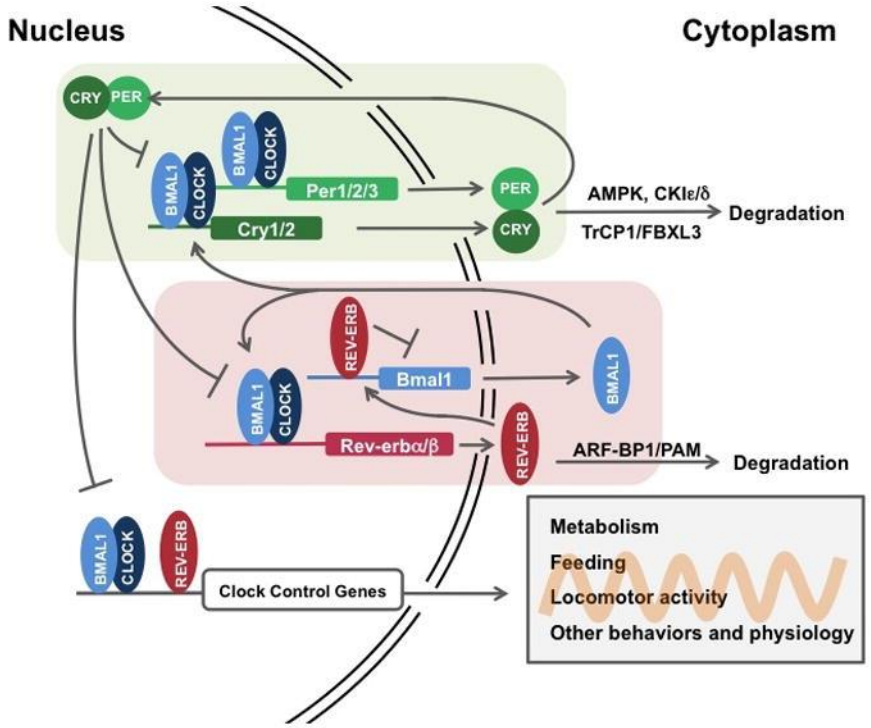


FIGURE 1.7: The basic clock machinery. The core clock consists of a series of transcriptional/translational feedback loops that synchronise diverse metabolic and physiological processes. BMAL1, Aryl hydrocarbon receptor nuclear translocator-like (ARNTL), CLOCK, circadian locomoter output cycles kaput; CRY1/2, cryptochrome 1/2; PER 1/2/3, period 1/2/3; AMPK, adenosine monophosphate-activated protein kinase; CKIε/ δ, by casein kinases ε/ δ; TrCP1, transducin repeat containing protein; ARF-BP1/ PAM; adenosine diphosphate-ribosylation factor-binding protein 1/ adenosine diphosphate-ribosylation factor-binding protein 1 / protein associated with Myc. Image adapted from (Feng and Lazar, 2012).

limiting step of the cholesterol biosynthetic pathway and its mRNA levels vary diurnally, reaching a peak during the dark phase (Brown et al., 1979). Similarly, the enzymes PEPCK and glucose-6-phosphatase are involved in hepatic glucose production and secretion, and are directly regulated by REV-ERBα (Yin et al., 2007).

Moreover, the core clock machinery can drive circadian expression of many transcription factors, thus extending and enhancing its regulatory function. Among these factors are the three members of the proline- and acidic amino acid-rich (PAR) basic leucine zipper proteins, albumin D site binding protein, thyrotroph embryonic factor (TEF), and (HLF), and the related protein E4BP4. All four of these proteins bind to D boxes, but while DBP, TEF, and HLF are activators, E4BP4 is a repressor. For instance, *in vivo* and *in vitro* studies have demonstrated that E4BP4 is a direct REV-αBα target in the liver; E4BP4 is a transcription factor which negatively regulates CYP7A1, thus reducing hepatic bile acid synthesis (Duez et al., 2008).

Furthermore, recent studies have suggested that the nuclear receptor superfamily in fact comprises a vast array of clock-controlled genes which present with a tissue-specific diurnal expression profiles, thus linking peripheral clock machinery to metabolic physiology. Specifically, a detailed study surveying the diurnal profiles of all 49 mouse nuclear receptors in key metabolic tissues has demonstrated that 25 of these ligand-activated transcription factors are expressed in a tissue-specific rhythmic manner (Yang et al., 2006). For example, CLOCK and BMAL1 can directly regulate PPARα expression via binding to an E-box element in its promoter region. At the same time, the promoter region of BMAL1 harbours a PPARα response element and thereby is a direct target of this transcription factor. This reciprocal regulation exemplifies the very close link between nuclear receptors, metabolism and the circadian clock. Circadian clock also regulates the mammalian transcriptome by modulating the stability and activity of nuclear receptors. Specifically, *in vitro* studies have shown that PER2 binds as a corepressor to estrogen receptor α (ERα) and targets it for proteosomal degradation (Gery et al., 2007). Moreover, PER2 is shown to directly associate with PPARγ and inhibit its function by blocking the recruitment of this nuclear to target promoters (Grimaldi et al., 2010). Furthermore, the circadian clock controls the transcriptional activity of nuclear receptors indirectly by modulating the availability of their specific endogenous ligands. For examples glucocorticoids are potent steroid hormones which are secreted by the adrenal glands in a distinct circadian pattern and are able to synchronise not only the activity of the glucocorticoid receptor itself but also approximately 60% of the circadian transcriptome (Reddy et al., 2007). These observations further support the notion that the circadian clock regulates metabolism at various levels.

1.6.4 Circadian disruption and metabolic disease in rodents

The role of circadian clocks in metabolic regulation is well supported by genetic evidence that mutation in clock genes disturbs rhythmic expression of key metabolic genes and causes metabolic disorders.

Lipid homeostasis is tightly controlled by the circadian system. Therefore, CLOCK-deficient mice develop obesity and severe metabolic syndrome (Turek et al., 2005). These animals are hyperphagic and show disrupted feeding rhythms correlating with impaired expression of energy-regulatory peptides (e.g. leptin) in the SCN (Turek et al., 2005). Mice lacking CLOCK have increased body weight, predominantly via fat deposition leading to adipocyte hypertrophy and adiposity (Turek et al., 2005). While some blood lipids such as triglycerides and cholesterol are increased in these mutants, the levels of NEFA and glycerol are significantly reduced (Turek et al., 2005). This effect is inconsistent with the observed metabolic phenotype and stems from local changes in adipose tissues. Specifically, deletion of CLOCK in adipocytes impairs triglyceride mobilisation by not only reducing the transcript levels but also phase-shifting the oscillatory patterns of ATGL and HSL (Shostak et al., 2013b). However, it has been proposed that in addition to WAT dysfunction, intestinal overabsorption of dietary fats (Pan and Hussain, 2009) and enhanced de novo lipogenesis in the liver (Pan et al., 2010) could also contribute to the development of the dyslipideamic phenotype in CLOCK mutant mice. Similarly, BMAL1-deficient mice present with increased adiposity and normal food intake suggesting the WAT dysfunction could play a role in this phenotype (Lamia et al., 2008, Shimba et al., 2011). Indeed, adipose tissue-specific disruption of BMAL1 reduces the content of polyunsaturated fatty acids in adipocytes by impairing the expression of key lipogenic genes (e.g. SCD1) (Paschos et al., 2012). This, together with a time-specific decrease in lipolysis, reduces the amount of plasma long-chain polyunsaturated fatty acids during the light phase thereby changing the expression of hypothalamic neuropeptides that regulate appetite and impairing feeding rhythms in rodents. Furthermore, both CLOCK mutation and BMAL1 deficiency promote ectopic lipid accumulation in liver and skeletal muscle (Turek et al., 2005, Shimba et al., 2011). Moreover, both REV-ERB isoforms are responsible for the regulation of *de novo* lipogenesis in the liver and therefore ablation of these two nuclear receptors results in severe hepatosteatosis (Bugge et al., 2012). Also, studies in REV-ERBα knockout mice and REV-ERBα overexpressing mice have demonstrated that this nuclear receptor mediates the cyclic transcription of Insulin induced gene 2 (INSIG2), encoding a trans-membrane protein that sequesters SREBP proteins to the endoplasmic reticulum membranes and thereby interferes with the proteolytic activation of SREBPs in Golgi membranes (Le Martelot et al., 2009). Thus, REV-ERBα modulates the diurnal activity of SREBP1c (key regulator of fatty acid biosynthesis) and SREBP2 (key regulator of cholesterol biosynthesis) and thereby controls the

circadian oscillations in cholesterol and fatty acid metabolism (Le Martelot et al., 2009). By inhibiting lipid biosynthesis and driving hepatic lipid storage, REV-ERBα reroutes gluconeogenic metabolites and indirectly promotes gluconeogenesis (Sun et al., 2012). Also, REV-ERB double knockout mice present with disturbed locomotor activity, increased circulating triglyceride and dysregulation of overall fuel metabolism of the body (Cho et al., 2012). In contrast, *in vivo* studies have shown that administration of synthetic REV-ERB agonists inhibit lipid and cholesterol synthesis in liver and fat, and promote fatty acid and glucose oxidation in muscle, resulting in increased energy expenditure (Solt et al., 2012). These compounds also significantly improve dyslipidemia and hyperglycaemia in diet-induced obesity models (Solt et al., 2012). Furthermore, REV-ERBα regulates cholesterol homeostasis by controlling bile acid synthesis. Specifically, it directly modulates the expression of CYP7A1, small heterodimeric partner (SHP) and E4BP4 and therefore the diurnal rhythmicity in distribution of their transcripts is lost in the absence of this clock gene (Duez et al., 2008). REV-ERBα-deficient mice present with reduced biosynthesis and impaired biliary and faecal excretion of bile salts (Duez et al., 2008).

Glucose homeostasis is under circadian control at the level of both peripheral tissues and the SCN. *In vivo* studies in rats have demonstrated that basal glucose levels peak and glucose tolerance is enhanced at the beginning of the active phase as compared to the resting phase (la Fleur et al., 2001). The morning peak of glucose is postulated to result from increased hepatic gluconeogenesis as well as low pancreatic insulin secretion, whereas the improved glucose tolerance (early in the active period) is likely due to elevated glucose uptake in skeletal muscle and adipose tissues (la Fleur et al., 2001). Genetic disruption of components of the clock network has identified a role of core clock genes in glucose homeostasis. For example, CLOCK-deficient mice present with impaired glucose tolerance, reduced insulin secretion and defects in size and proliferation of pancreatic islets that worsen with age (Damiola et al., 2000). CLOCK disruption in the pancreas not only reduces the transcript levels but also phase-shifts the oscillatory patterns of pancreatic genes pivotal for insulin signalling, glucose sensing as well as islet growth and development, thereby leading to hyperglycaemia (Marcheva et al., 2010). Similarly, pancreas-specific BMAL1 deletion interferes with the diurnal variation in the expression of key genes in this tissue which results in reduced insulin secretion, impaired glucose tolerance and hyperglycaemia throughout the whole light-dark cycle (Marcheva et al., 2010). On the other hand, loss of this core clock component in the liver abolishes rhythmic expression of glucose metabolic genes (e.g. PEPCK and GLUT2) and leads to hypoglycaemia only in the fasting phase of the day (Lamia et al., 2008). Also, CRY1 is involved in the circadian regulation of hepatic gluconeogenesis. Specifically, CRY1 expression is elevated during the night-day transition when it reduces fasting gluconeogenic gene expression by blocking glucagon-mediated increases in intracellular cAMP concentrations and in the PKA-

mediated phosphorylation of cAMP response element-binding protein (CREB) (Zhang et al., 2010a). Therefore, hepatic depletion of CRY1/2 increases circulating glucose while CRY1 overexpression reduces fasting blood glucose and improves whole body insulin sensitivity in genetically-obese mice (Zhang et al., 2010a). CRYs also transrepress glucocorticoid-induced PEPCK transcription, therefore loss of CRYs results in glucose intolerance (Lamia et al., 2011). Similarly depletion of both REV-ERB isoforms increases fasting blood glucose (Cho et al., 2012).

1.6.5 Circadian disruption and metabolic disease in humans

The profound impact of circadian rhythmicity on metabolism in humans is illustrated by considerable epidemiological evidence that night-shift workers and individuals with sleep disorders present with increased risk for obesity, diabetes, and cardiovascular disease as a result of physiologic maladaptation to chronically sleeping and eating at abnormal circadian times (Esquirol et al., 2009). Moreover, a recent human study has demonstrated that misalignment between behavioural cycles (fasting/feeding and sleep/wake cycles) and endogenous circadian cycles results in systemically reduced leptin concentrations, decreased insulin sensitivity, hyperglycaemia and abnormal diurnal patterns of cortisol secretion (Scheer et al., 2009). Additionally, circadian misalignment also increases mean arterial pressure and diminishes sleep efficiency thereby predisposing to the development of cardiometabolic disease (Scheer et al., 2009). Furthermore, plasma triglycerides are reportedly raised in shift workers and this association is independent of the levels of physical activity and dietary habits (Esquirol et al., 2009). Increased lipogenesis in combination with elevated food consumption and reduced energy expenditure, as consequence of hypoleptineamia, could facilitate the development of obesity in individuals suffering from circadian desynchrony. These observations emphasise the intimate connection between circadian function and metabolic homeostasis.

1.6.6 Circadian rhythmicity in pregnancy

Pregnancy is a physiologically challenging state and has been shown to impact the expression of clock genes in maternal tissue. In particular, it has been demonstrated that during rat pregnancy there are alterations in the rhythmic profile of PER2 in the maternal brain (Schrader et al., 2010, Schrader et al., 2011). Moreover, studies in mouse pregnant liver suggest that there is increased circadian oscillation of PER3 while the diurnal rhythmicity of CLOCK, BMAL1, PER1, CRY1 and CRY2 is dampened (Waddell et al., 2012). It has been proposed that since clock genes are expressed in uterine tissue (Akiyama et al., 2010) they could play a role in the timing of parturition in humans and rodents.

Furthermore, recent studies have reported that clock genes are expressed in rodent placenta and also exhibit diurnal oscillation of low amplitude (Ratajczak et al., 2010, Wharfe et al., 2011). Interestingly, morphologically and functionally distinct regions in rat placenta (labyrinth and junctional zones) appear to have differential expression of clock genes (Wharfe et al., 2011). In the labyrinth zone, CLOCK, PER1 and CRY2 expression is higher compared to the junctional zone, while the opposite tendency is observed for BMAL1, PER2, PER3 and CRY1 (Wharfe et al., 2011). It has been further observed that the expression of BMAL1, PER1 and PER2 peaks during the night and does not follow an anti-phase pattern, suggesting that the transcriptional regulation of placental clock genes could be fundamentally different (Wharfe et al., 2011).

Also, studies in mice have shown that genetic disruption of the circadian clock could influence negatively reproductive function and pregnancy outcomes. It has been reported that CLOCK-deficient mice present with irregular estrous cycles, enhanced fetal reabsorption and increased rate of full-term pregnancy failure (Miller et al., 2004). Similarly, BMAL1 knockout mice have increased risk of miscarriage as well as disturbed estrous cycling (Kennaway, 2005). Moreover, deletion of BMAL1 in mouse ovaries impairs the activity of the core clock machinery and also disturbs the expression of key genes involved in steroidogenesis, therefore these animals present with embryonic implantation failure as a consequence of progesterone deficiency (Liu et al., 2014).

Additionally, a recent study examining the impact of intrauterine environment on fetal health has demonstrated that circadian disruption during primate pregnancy causes desynchronisation and overall reduction in the body temperature of the newborn; exposure of pregnant monkeys to constant light does not have any impact on maternal body temperature rhythm (Seron-Ferre et al., 2013). These data strongly suggest that exposure of the fetus to circadian insult *in utero* could have an adverse effect on its energy metabolism and hormonal signalling, both of which control core body temperature.

Finally, studies in female shift-workers and women who travel often across time zone suggest that circadian disruption could result in altered menstrual function (Labyak et al., 2002), reduced fertility (Bisanti et al., 1996) as well as adverse pregnancy outcomes, including increased incidence of low birth weight, preterm delivery and miscarriage (Mahoney, 2010).

In conclusion, circadian rhythms play a key role in the regulation of lipid and carbohydrate metabolism as well energy homeostasis. However, there is a substantial gap in our knowledge regarding the impact of pregnancy on peripheral clocks in key metabolic tissues of the mother. Also, it not clear whether the core clock machinery, previously described in the placenta, controls the metabolic function of this organ and thereby affects fetal development by

modulating the transsyncytial transport of essential nutrients and metabolic substrates. Part of the work presented in this document will aim to expand the knowledge on these topics.

1.7 Hypothesis

We hypothesise that several metabolic pathways change during pregnancy and thereby cooperatively orchestrate the gestational adaptations in maternal lipid homeostasis. Specifically, LXR signalling is altered and this contributes to the switch in lipid metabolism during this period. Moreover, metabolic pathways in subcutaneous and visceral WAT are differentially regulated in pregnancy and gestational cholestasis, and this is conducive to the alterations in lipid homeostasis. Furthermore, maternal adaptations in energy homeostasis during pregnancy result also from the altered diurnal fluctuations in peripheral metabolic pathways.

1.8 Aims

Study the changes in lipid homeostasis during murine pregnancy

Investigate the contribution of LXR signalling to maternal adaptations in lipid metabolism

Examine the impact of pregnancy on the structure, metabolic activity and endocrine profiles of subcutaneous and visceral fat

Determine whether the structure, metabolic activity and endocrine profiles of subcutaneous and visceral fat depots are altered in pregnancies complicated with gestational cholestasis

Study the impact of pregnancy on the expression of peripheral oscillator components in key metabolic tissues

Examine the impact of pregnancy on the diurnal oscillations of key metabolic pathways in peripheral tissues

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Manufacturers

All materials and solvents used were of analytical grade and were purchased from one of the following companies: Alpha Laboratories (Eastleigh, UK); Abcam (Cambridge, UK); Applied Biosystems (Cheshire, UK); Bio-Rad (Hemel Hempstead, UK); BMG LABTECH (Aylesbury, UK); Cambridge Biosciences (Cambridge, UK); Cayman Chemicals Michigan, USA); Cell Signalling Technology (Danvers, USA); Corning (New York, USA); Dako (Ely, UK); Diagenode (Cambridge, UK); Eppendorf (Stevenage, UK); Fisher Scientific (Loughborough, UK); GE Healthcare (Piscataway, USA); GraphPad Software (La Jolla, USA); Greiner Bio-One (Stonehouse, UK); Qiagen (West Sussex, UK); Invitrogen (Paisley, UK); IGP (Essex, UK); LBS Biotechnology (Horley, UK); Makaira Ltd (London, UK); Millipore (Watford, UK); Nikon UK Limited (Surrey, UK); Perkin Elmer (Seer Green, UK); Promega (Southampton, UK); Qiagen (Craley, UK); Roche Applied Science (Indianapolis, USA); Santa Cruz (Heidelberg, Germany); Severn Biotech (Worcestershire, UK); Sigma-Aldrich (Peterborough, UK); StarLab (Milton Keynes, UK); Thermo Fisher Scientific (Lutterworth, UK); VWR (Lutterworth, UK); Wilmington (Delaware, USA).

2.1.2 Chemicals and Reagents

Acrylamide-Bis-acrylamide 30%	Sigma-Aldrich
Ammonium Persulphate	Sigma-Aldrich
β-mercaptoethanol	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma-Aldrich
Chloroform	Sigma-Aldrich
Cholic acid	Sigma-Aldrich
Collagenase Type 2	Sigma-Aldrich
DEPC-treated water	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma-Aldrich
dNTP Mix, 10mM	Invitrogen
Ethanol	VWR
Entellan	Millipore
Ficoll- Paque Plus	GE Healthcare
Glycine	Sigma Aldrich
GW3965	Sigma-Aldrich
Hank’s Balanced Salt Solution	Sigma-Aldrich

Harris Modified Heamatoxylin Solution	Sigma-Aldrich
Isopropanol	Sigma-Aldrich
Luminata Classico Western HRP substrate	Millipore
Luminata ForteWestern HRP substrate	Millipore
Lane Marker Reducing Sample Buffer, 5X	Thermo Scientific
Methanol	VWR
NP40 Detergent	Abcam
Phopshate Buffered Saline (PBS)	Sigma-Aldrich
Phosphatase Inhibitors Cocktails 2 and 3	Sigma-Aldrich
Ponceau Red Stain	Sigma-Aldrich
Protease Inhibitors Cocktail	Sigma-Aldrich
QIAzol	Qiagen
RIPA Buffer	Sigma-Aldrich
Restore Western Blot Stripping Buffer	Thermo Scientific
SeeBlue Plus2 Pre-Stained Standard	Invitrogen
SYBR Green JumpStart Taq ReadyMix for quantitative PCR	Sigma-Aldrich
Skim Milk Powder	Sigma-Aldrich
Sodium dodecyl phosphate (SDS)	Sigma-Aldrich
Saline Solution, 0.9%	Severn Biotech
TEMED	Sigma-Aldrich
T0901317	Cayman Chemicals
Tris-Base	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Tween 20	Bio-Rad

2.1.3 Kits

Mouse Adipokine Antibody Array	R & D Systems
RNaeasy Mini Kit	Qiagen
RNeasy Lipid Tissue Mini Kit	Qiagen
RNeasy Micro Kit	Qiagen

Pierce BCA Protein Assay Kit	Thermo Scientific
Supercript II Reverse Transcriptase Kit	Sigma-Aldrich
Wako LabAssay Triglycerides kit	Alpha Laboratories

2.1.4 Antibodies

Anti-LXR alpha antibody (ab41902), 1:1000	Abcam
Anti-LXR beta antibody (ab28479) 1:500	Abcam
Anti-GAPDH antibody (MAB374), 1:1000	Millipore
Goat anti-mouse IgG-HRP (sc-2031), 1:4000	Santa Cruz
Goat anti-rabbit IgG-HRP (P0448), 1:2000	Dako

2.1.5 Animal Diets

Rat and Mouse No3 (RM3) standard breeding diet	Special Diet Servicing
Rat and mouse CRM (P) standard breeding diet	Special Diet Servicing
RM3 diet supplemented with 0.012% T0901317	LBS Biotechnology
CRM diet supplemented with 0.5% cholic acid	LBS Biotechnology

2.1.6 Buffer Recipes

Blocking Buffer	TBS-T buffer, 5% (w/v) BSA or 5% (w/v) skim milk powder
Sodium Citrate Buffer, 0.38%	0.9% Saline Solution, 0.38% (w/v) Sodium Citrate pH 7.4
NP40 Buffer	ddH_20, 5% (w/v) NP40
SDS Buffer (10x)	250mM Tris-Base, 1.9M Glycine, 1% (w/v) SDS

Transfer Buffer (10x)	25mM Tris-Base, 192mM Glycine, 20% (v/v) Methanol pH 8.3
Tris Buffered Saline (TBS) (1x)	20mM Tris, 150mM NaCl pH 7.6
Tris Buffered Saline, 0.05% Tween 20 (TBS-T) (1x)	1x TBS, 0.05 (v/v) Tween 20
Triton X-100 Lysis Buffer	0.125M KH_2PO_4, 1mM EDTA, 0.1% (v/v) Triton X-100 pH 7.4

2.1.7 Miscellaneous

Amersham Hyperfilm ECL	GE Healthcare
Bioruptor Plus system	Diagenode
EDTA-coated tubes	Fisher Scientific
GraphPad Prism 6	GraphPad Softaware
Light Miscroscope	Nikon
LX20 autoanalyzer	Beckman Coulter
MicroAmp Optical 384-Well Reaction Plate	Applied Biosystems
Nanodrop ND-1000 spectrophotometer	Wilmington
Nitrocellulose membrane, 3mm	Bio-Rad
Novex Gel Cassette mini 1.5 mm	Invitrogen
Novex NuPAGE Gel System	Invitrogen
PHERAstar FS microplate reader	BMG LAMBETH
PVDF membrane	Bio-Rad
TissueLyser II system	Qiagen
ViiA 7	Applied Biosystems
XCell SureLock Mini-Cell Electrophoresis System	Invitrogen
Whatman filter paper	GE Healthcare

2.2 Methods

2.2.1 In vivo Mouse Studies

2.2.1.1 General maintenance

Wild type C57BL/J6 mice were purchased from Harlan Laboratories. Some of animals were maintained at the animal facilities of Central Biomedical Services (CBS), Imperial College London in a light/dark (12h/12h) cycle (lights on 7:00-19:00) with free access to standard chow diet (RM3). The rest of the mice were maintained at the animal facilities of Biological Services Unit (BSU), King's College London in a light/dark (12h/12h) cycle (lights on 7:00-19:00) with free access to standard chow diet (CRM). All studies were conducted in accordance with The Animals Scientific Procedures Act 1986 and were approved by local animal ethics committees and were authorised by the Home Office.

2.2.1.2 T0901317 feeding in pregnancy experiment

This experiment was conducted on the premises of CBS, Imperial College London. Age-matched (6-8 week old) female C57BL/6 mice were mated with age-matched C57BL/6 males (8-10 week old) in a 2:1 ratio (2 females and 1 male per cage). Females were visually inspected for the presence of a copulation plug daily and, following the identification of one, the female mice were separated from the males and were fed standard RM3 diet or RM3 diet supplemented with 0.012% T0901317 (Schultz et al., 2000) (T0901317 diet). Pregnant females were sacrificed on days 7, 10, 14 and 18 of gestation. Tissues were collected from RM3-fed virgin controls and non-pregnant mice fed with a T0901317 diet for a period of 7, 10, 14 or 18 days. Mice, fasted for 4 hours with free access to water, were sacrificed by CO_2 asphyxiation. Tissues of interest were collected and weighed. All tissues were snap frozen in liquid nitrogen or fixed in formalin for 24 hours for histological analysis and then transferred into PBS.

2.2.1.3 Cholic acid feeding in pregnancy experiment

This experiment was conducted on the premises of BSU, King's College London. Age-matched (6-8 week old) female C57BL/6 mice were mated with age-matched C57BL/6 males (8-10 week old) in a 2:1 ratio (2 females and 1 male per cage). Females were visually inspected for the presence of a copulation plug daily and following the identification of one, the female mice were separated from the males and were fed standard CRM diet or CRM diet supplemented with 0.5% cholic acid (CA diet; previously described by (Watanabe et al., 2006, Papacleovoulou et al., 2013)). Pregnant females were sacrificed on day 14 of gestation. Tissues were collected

from CRM-fed virgin controls and non-pregnant mice fed with a CA diet for a period of 14 days. Mice, fasted for 4 hours with free access to water, were sacrificed by CO_2 asphyxiation. Tissues of interest were collected and weighed. All tissues were snap frozen in liquid nitrogen or fixed in formalin for 24 hours for histological analysis.

2.2.1.4 Diurnal metabolic fluctuations in pregnancy experiment

This experiment was conducted on the premises of CBS, Imperial College London. Age-matched (6-8 week old) female and male C57BL/6 mice were allowed to acclimatise for a period of 2 weeks and then were coupled in 1:1 ratio (1 female and 1 male per cage). Females were visually inspected for the presence of a copulation plug daily. Pregnant animals were collected on days 7 and 14 of pregnancy. As previously described (Milona et al., 2010), non-pregnant controls were animals sacrificed one day after the identification of the copulatory plug (day 2 of pregnancy) since they were expected to be at the same stage of their menstrual cycles at that point. Non-pregnant and pregnant animals were sacrificed by cervical dislocation at 4 hour intervals over a period of 24 hours with 5-8 animals per group. During the dark phase, animals were collected under infrared light. Tissues of interest (liver, gonadal adipose tissue, skeletal muscle, terminal ileum, placenta and fetal liver (from day 14 pregnant mice) were dissected and snap frozen in liquid nitrogen.

2.2.1.5 Metabolic monitoring of pregnant mice

This experiment was conducted on the premises of CBS, Imperial College London. Age matched (6-8 week old) female and male C57BL/6 mice were mated and then the females were visually inspected for the presence of a copulation plug on daily basis. The Comprehensive Laboratory Animal Monitoring System (CLAMS, Columbus Instruments, Columbus, OH) is a set of live-in cages for automated, non-invasive and simultaneous monitoring of VO_2 (volume of oxygen consumed, mL/kg/hr), VCO_2 (volume of carbon dioxide produced, mL/kg/hr), RER (respiratory exchange ratio), heat (kcal/hr), accumulated food (grams), accumulated drink (grams). Day 16 pregnant animals were individually placed in CLAMS cages and monitored over a 3 day period. The raw data was analysed using CLAX statistical software as per manufacturer's instructions.

2.2.2 Isolation of primary murine cells

2.2.2.1 Isolation of peripheral blood monocyte cells (PBMCs)

Murine blood was collected in EDTA-coated tubes (to prevent clotting) and was kept on ice. Following a 10 minute centrifugation at 10000rpm at 4°C, the serum fraction was removed and stored at -80°C for subsequent analysis. The plasma fraction was gently resuspended in 3mL of 0.38% citrate buffer and then centrifuged at room temperature for 10 minutes at 800rpm. The supernatant was aspirated and the pellet was resuspended in 5mL of 0.38% citrate buffer. 3mL of Ficoll-Paque Plus were added to a 15mL tube. The diluted plasma was layered very slowly on top of Ficoll-Paque Plus using a Pasteur pipette with the tube held at a 45° angle. Care was taken to avoid mixing of the blood sample and the polysaccharide solution. The sample was centrifuged at 400rcf for 35 minutes at 20°C without a brake. The ring-like cellular aggregate was gently removed and transferred to a clean tube. 1mL of QIAzol was added to the cells and, following intense vortexing, the cellular suspension was either directly subjected to RNA extraction using an RNeasy Micro Kit or stored at -80°C.

2.2.2.2 Isolation of primary adipocytes

Adipose tissue was dissected and placed in chilled PBS on ice. The tissue was thoroughly minced with scissors in a weighing boat on ice. The minced tissue was transferred into a 50mL tube containing 3mL of chilled collagenase buffer (3mg of Collagenase Type 2 per 1mL of PBS). The tubes were incubated at 37°C for 1 hour, vigorously shaking at 120rpm. After the digestion was completed, the cell suspension was passed through a 100µm sieve to remove undigested fragments. The cell slurry was centrifuged at 500rcf for 10 minutes at 4°C to separate the adipocyte fraction (floating on top) and the stromal vascular fraction (pelleted at the bottom of the tube). The stromal vascular fraction was aspirated (care was taken not to disturb the floating adipocytes), transferred into a 2mL tube, then thoroughly mixed with 1mL of QIAzol and stored at -80°C for subsequent analysis. The adipocyte fraction was also lysed with 1mL of QIAzol following which the cell suspension was either subjected to RNA extraction using RNeasy Lipid Tissue Mini Kit or was stored at -80°C for subsequent analysis

2.2.3 Gene expression analysis

2.2.3.1 Extraction of RNA from cells and tissues

Total RNA extraction was performed using RNeasy Mini Kit (cells, liver and placenta samples) or RNeasy Lipid Tissue Mini Kit (adipose tissue, skeletal muscle and duodenum) or RNeasy

Micro Kit (PBMC). To isolate RNA from tissues, frozen segments were homogenised in the appropriate lysis buffer (RLT buffer supplemented with β-mercaptoethanol for liver and placenta, and QIAzol lysis reagent for muscle, adipose tissue, duodenum and peripheral blood monocytes) using TissueLyser II system for 2 minutes at 30Hz. The homogenates were then processed in accordance with the manufacturer's protocol. Purified RNA was re-suspended in RNase-free water and RNA concentrations were measured by a Nanodrop spectrophotometer. An OD260/ OD280 ratio of more than 2 was considered indicative of good purity RNA. RNA samples were stored at -80^{o} C.

2.2.3.2 cDNA synthesis

1µg of total RNA was reverse transcribed using SuperScript II Reverse Transcriptase in accordance with the manufacturer's protocol. In order to test for the presence of genomic DNA a negative control was included in which the SuperScript II enzyme was replaced with 1µL of DEPC-treated water (RT-negative samples). All cDNA samples were diluted with 150µL of DEPC-treated water and were stored at -80^{o} C.

2.2.3.3 Amplification of target cDNA

Real-time quantitative PCR (RT-PCR) assays were performed using a Viia7 system. Genes of interest were amplified in duplicate using SYBR Green Mastermix. The assays were prepared in a 384-well format and each reaction contained 4µL of SYBR Green, 1µM of forward/reverse primer (relevant primer sequences are listed in Appendix I) and 3.2µL of cDNA. Negative controls were reactions using the RT-negative sample as well reactions amplifying 3.2µL of DEPC-treated water instead of cDNA. All assays were performed under the following thermal cycler conditions: rapid initial heating to 95^{o}C for 2 minutes, denaturation at 95^{o}C for 15 seconds and annealing/extension at 60^{o}C for 60 seconds for a total of 40 cycles. To ascertain that all cDNA samples produced by this method were of equal quality, we tested the abundance of a housekeeping gene (cyclophilin for mouse samples). This gene was also used for downstream analysis (see below).

2.2.3.4 Comparative method of threshold gene expression quantification

Comparative Ct method for quantification uses arithmetic calculations to determine the relative expression of a given gene in a sample of interest by comparing it to a housekeeping gene and calibrator sample. The method is based on the assumption that difference in the threshold cycles (ΔCt) between the gene of interest and the housekeeping gene is proportional to the expression level of the gene of interest (ΔCt = Ct (gene of interest) –Ct (cyclophilin) (Livak and

Schmittgen, 2001). Next, the normalised expression of the target gene in the sample of interest relative to its expression in the calibrator sample is calculated ($\Delta\Delta Ct = \Delta Ct$ (sample) - ΔCt (calibrator)). Finally, the fold change in expression is given by $2^{\Delta\Delta Ct}$.

2.2.3.5 RT-PCR primer design

Gene-specific primers were designed in order to amplify a short sequence of cDNA producing a product of approximately 100bp which crosses intron-exon boundaries. Gene sequences were obtained from the Ensembl database and were analysed by PerlPrimer software version 1. 1. 21. Primers were re-suspended using DEPC-treated water to a stock concentration of 100μM and diluted to a working concentration of 20 μM. All primers were stored at -20^{o} C. The amplification efficiency of the primers was validated by template titration curves and temperature dissociation analyses. The nucleotide sequences of relevant primers are listed in the Appendix I.

2.2.4 Protein analysis

2.2.4.1 Extraction of proteins from tissues

To extract proteins, frozen tissue fragments were homogenised in RIPA buffer supplemented with a protease inhibitor cocktail as well as phosphatase inhibitor cocktails 2 and 3 using TissueLyser II system for 2 minutes at 30Hz. The lysates were then sonicated for 2 minutes at 4^{o}C (a 30 seconds sonication phase followed by a 30 seconds resting phase) using a Bioruptor Plus system. The protein lysates were centrifuged at 13,000 rpm for 20 minutes at 4^{o} C following which the supernatants were transferred to clean tubes and stored at -80^{o} C.

2.2.4.2 Protein quantification

Colorimetric detection and quantification of proteins was conducted using Pierce BCA Protein Assay Kit according to the manufacturer's instructions. The assay was run in a sample to working reagent ratio of 1:50 (v/v) format and was analysed by measuring absorbance (562nm wavelength) using a PHERAstar FS microplate reader. Since the assay provides relative quantification, all samples relevant to a given experiment were analysed simultaneously. Depending on how concentrated the protein lysates were, aliquots of the samples were quantified following a dilution with RIPA buffer (1:10 dilution was usually used).

2.2.4.3 SDS polyacrylamide gel electrophoresis

Discontinuous polyacrylamide gel electrophoresis was used to separate proteins based on molecular mass using a Novex NuPAGE Gel System. Briefly, the stacking and resolving gels were prepared following the recipe provided below.

TABLE 2.1: Stacking and Resolving Gel Recipe

Component	Stacking Gel	12% Resolving Gel
ddH_2O [mL]	3.675	2.1
1M Tris-HCL pH 6.8 [mL]	0.625	-
1M Tris-HCL pH 8.8 [mL]	-	3.75
10% SDS [µL]	50	100
Acrylamide-Bis-acrylamide 30% [mL]	0.670	4
10% Ammonium persulphate [µL]	25	50
TEMED [µL]	5	5

Following its preparation, the resolving gel solution was poured into a Novex Gel Cassette mini 1.5 mm, then overlaid with 1mL of Isopropanol and allowed to set at room temperature for approximately 40 minutes. Once the polymerisation was complete, the isopropanol was removed and the surface of the gel was rinsed with ddH_2O then dried of any excess liquid with Whatman filter paper. The stacking gel solution was then poured on top of the resolving gel and the appropriate comb (10 or 15-well comb) was inserted. Once the polymerisation was complete the gel was either used immediately or was stored overnight at 4^oC wrapped in wet tissues and transparent foil in order to prevent drying. Prior to the loading of the samples, the wells of the gel were rinsed with 1x SDS buffer to remove any unpolymerised acrylamide. Protein samples containing 40µg of protein and SDS loading buffer diluted to 1x concentration were boiled at 95^oC for 5 minutes and then spun down. The resulting denatured samples as well as the molecular weight marker SeeBlue Plus2 Pre-Stained Standard were loaded on a gel and run at a constant voltage of 80V for approximately 30 minutes. Once the samples entered the resolving gel, the voltage was increased to 110V and was maintained until the loading dye had migrated to the bottom of the gel. Next, the gel was immediately removed from the cassette and subjected to Western blotting.

2.2.4.4 Western blotting

Following their separation based on molecular mass, the proteins were transferred to a membrane using an electric field oriented perpendicular to the surface of the gel, causing the peptides to migrate out of the gel and then onto the membrane. Wet blotting method was employed to transfer the proteins at 30V for 120 minutes using the XCell II Mini Blot Module system in accordance with the manufacturer's instructions. PVDF and nitrocellulose membranes were used depending on the properties of the antibodies (for the detection of LXRα and LXRβ, the protein samples were transferred to a nitrocellulose membrane). The membrane was then stained with Ponceau Red in order to visually confirm the presence of proteins on the membrane as well the absence of bubbles which could potentially interfere with the immunoblotting. The membrane was then incubated with gentle rotation in blocking buffer for 1 hour at RT. Next, the membrane was incubated with the primary body solution (listed in section 2.1.5) at 4^{o} C overnight (this incubation step may vary depending on the properties of the antibody). Following, a series of 3 washes with 1xTBS-T buffer for 5 minutes at RT, the membrane was incubated with horse-radish peroxidase (HRP) conjugated secondary antibody (refer to 2.1.5) for 1 hour at room temperature. Following a series of 5 washes with 1x TBS-T buffer for 5 minutes at room temperature, the membranes were incubated with peroxidase substrate at room temperature. Peroxidase activity was visualized via enhanced chemiluminescence on exposure hyperfilms and the blots were developed by an X-ray film processor. Densitometric analysis of bands was performed using ImageJ software.

2.2.4.5 Stripping of antibodies from Western blots

To re-probe previously immunoblotted membranes, the antibodies were stripped using Restore Western Blot Stripping Buffer according to the manufacturer's instructions. The membrane was then incubated with blocking buffer and probed with antibodies as described in 2.2.4.4.

2.2.4.6 Mouse adipokine array

The protein levels of adipokines in murine white adipose tissue samples were quantified using a Mouse Adipokine Antibody Array kit in accordance with the instructions of the manufacturer. Following the preparation of the adipose tissue lysates (in agreement with the protocol provided by the manufacturer), 80mg of each protein sample (6 mice per group, 480mg of protein in total) were pooled and processed as a single sample. Pixel densities on developed X-ray films were measured and analysed using ImageJ software.

2.2.5 Lipid analysis

2.2.5.1 Extraction and quantification of lipids from liver, muscle, placenta

Lipid extraction was performed as previously described (Papacleovoulou et al., 2013). Briefly, frozen tissue fragments were homogenised in Hank's Balanced Salt Solution using TissueLyser II system for 2 minutes at 30Hz and then centrifuged the samples at 12000 rpm for 15minutes at 4^{o} C. The supernatant was discarded and the pellet was then re-suspended in 500μL of Triton-X100 lysis buffer. The samples were then sonicated for 8 minutes at 4^{o} C (a 30 seconds sonication phase followed by a 30 seconds resting phase) using a Bioruptor Plus system. Following a centrifugation at 10000rpm for 20 minutes at 4^{o}C, the supernatants were transferred to clean glass containers and were stored at -80^{o} C.

Serum and tissue extracts were processed and tested in the lab of Dr Eugene Jansen (Laboratory for Health Protection Research, National Institute of Public Health and the Environment, Bilthoven, Netherlands). Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and free fatty acids were measured using an LX20 autoanalyser.

2.2.5.2 Extraction and quantification of triglyceride from white adipose tissue

Approximately 30mg adipose tissue pieces were cut and weighed precisely. Each tissue fragment was thoroughly homogenised in 1mL of NP40 buffer using TissueLyser II system for 20 minutes at 30Hz. The samples were boiled for 5 minutes at 95-100°C or until the buffer became cloudy. After the samples were cooled down to room temperature, the heating step was repeated. The triglyceride content of each sample was measured using a Wako LabAssay Triglycerides kit in accordance with the instructions of the manufacturer. Total adipose tissue triglyceride content was calculated after normalising the measured triglyceride concentration of each sample to the exact weight of the starting fat fragment.

2.2.6 Histological examination of white adipose tissues

2.2.6.1 Tissue embedding and sectioning

Freshly harvested tissues were fixed in 10% neutral buffered formalin for up to 24 hours and were stored in PBS at room temperature until embedding. Tissues were then transferred into embedding cassettes and were washed under running water for 6 hours prior to dehydrating them in 70% ethanol overnight. Next, the tissues were further dehydrated by consequently immersing them in 70% ethanol (2 washes, 2 hours each), 95% ethanol (2 washes, 2 hours each)

and 100% ethanol (2 washes, 2 hours each). Samples were then "cleared" in xylene (2 washes, 2 hours each) to dissolved tissue lipids and then placed in melted paraffin wax (2 washes, 2 hours each) before embedding. All of the washing steps were performed using Leica Embedding System.

A microtome (Leica) was used to cut 5μm sections, which were mounted on Polysene-coated adhesion slides and incubated at room temperature overnight.

2.2.6.2 Haematoxylin and eosin staining

To melt the wax, slides were incubated at 60°C for 4 hours. The haematoxylin and eosin staining was performed following the steps listed below:

2 washes in xylene, 5 minutes each
1 wash in 100% ethanol for 5 minute
1 wash in 95% ethanol for 5 min
1 wash in PBS 10 min under constant stirring
Samples were rinsed running tap water for 30
Samples were incubated in haematoxylin for 2 min
Samples were rinsed under running tap water until excess blue dye was removed
Samples were subjected to 6 immersions in 0.3% acid alcohol
Samples were rinsed under running tap water until excess blue dye was removed
Samples were stained in eosin for 30 seconds
Slides were washed in 95% ethanol for 1 minute; this step was repeated twice
Slides were washed in 100% ethanol for 1 minute; this step was repeated twice
Slides were washed in xylene for 1 minute; this step was repeated twice

Cover-slips were mounted using Permount medium and the samples were examined using light microscopy.

2.2.8.3 Adipocyte area measurements

Images of haematoxylin-and-eosin-stained white adipose tissue sections were analysed using ImageJ software to determine the area of the separate adipocytes. Approximately 300-400 adipocytes were measured per tissue sample, 6 mice per group.

2.2.7 Statistical Analysis

All values were expressed as mean +/- SEM for biological replicates. Unpaired two-tailed t-test was used for single comparisons while multiple comparisons were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

For the analysis of diurnal fluctuation experiments (Chapter 5), differences between pregnant (gestational days 7 and 14) and non-pregnant mice at individual time points were assessed by one-way ANOVA. Circadian variation in gene expression and lipid abundance was also analysed using one-way ANOVA (Wharfe et al., 2011).

All statistical analyses were performed using GraphPad Prism 6 software package.

Chapter 3

Contribution of Liver X Receptor to the Adaptations in Maternal Lipid Metabolism during Pregnancy

3.1 Introduction

Pregnancy is a complex state characterised by alterations in maternal nutrient metabolism; these adaptations ensure that essential metabolites are continuously supplied to the growing fetus and also that the mother is provided with sufficient energy stores to meet the demands of pregnancy and prepare for lactation (Herrera, 2002). Lipid metabolism changes progressively throughout gestation and in the third trimester the mother develops hyperlipidaemia defined by increases in the concentrations of total and lipoprotein-bound cholesterol and triglycerides (Alvarez et al., 1996).

Lipid metabolism is regulated by the Liver X receptors (LXRs), which are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Apfel et al., 1994, Willy et al., 1995). LXR "senses" high levels of oxidised cholesterol derivatives and consequentially modulates the transcription of panoply of genes whose products protect cells from cholesterol-induced cytotoxicity (Lehmann et al., 1997). LXR plays an encompassing role in the regulation of cholesterol homeostasis: upon activation, it drives the efflux of cholesterol from non-hepatic tissues (e.g. macrophages and enterocytes), stimulates biliary excretion of the sterol in the liver, reduces the intestinal absorption of luminal sterols and downregulates cholesterol biosynthesis (Calkin and Tontonoz, 2012). LXR also stimulates *de novo* lipogenesis which facilitates cholesterol esterification (Schultz et al., 2000); this pathway is crucial for the protection of cells from the adverse effects of free cholesterol overaccumulation since it limits the solubility of the sterol in membrane lipids and promotes its sequestration into cytoplasmic lipid droplets (Nelson and Cox, 2013c).

However, there is a significant gap in our understanding of how LXR functions in the physiological setting of pregnancy. We hypothesised that the activity of LXR changes during pregnancy and this contributes to the development of gestational hypercholesterolemia. This chapter will focus on the gestational adaptations in murine lipid metabolism and also the impact of pregnancy on cholesterol homeostatic pathways that are governed by LXR. The impact of pharmacological activation of LXR on the pregnancy-induced alterations in lipid metabolism will also be detailed.

3.2 Experimental methodology

3.2.1 Adaptations in lipid metabolic pathways during mouse pregnancy

To study the gestational adaptations in lipid metabolism, mice were examined at different stages of pregnancy: pre-pregnancy, day 7 post coitum (pc), corresponding to early post-implantation pregnancy; day 10pc, corresponding to mid-pregnancy when placental layers have been defined; day 14 pc, representing advanced pregnancy when the fetoplacental unit undergoes active growth and development; day 18pc, corresponding to late pregnancy when the fetus continues to grow and the mother prepares for labour; virgin female mice were used as non-pregnant controls (Figure 3.1A and B) (Croy et al., 2014). Maternal serum, liver, duodenum and peripheral blood monocytes (PBMCs) were collected and analysed using molecular biology techniques (Figure 3.1B).

3.2.2 Impact of Pharmacological LXR Activation during Pregnancy on the Gestational Adaptations in Lipid Metabolism

To study the impact of pharmacological activation of LXR during mouse pregnancy on the gestational adaptations in lipid metabolism, female mice were fed with a standard chow diet supplemented with 0.012% T0901317 (Schultz et al., 2000) (T0901317 diet) following the identification of a copulatory plug (Figure 3.1C). Chow- and T0901317-fed pregnant mice were sacrificed on days 7, 10, 14 and 18 of gestation. Chow-fed virgin females and non-pregnant mice fed with a T0901317 diet for a period of 7, 10, 14 or 18 days were used as controls. Sera and tissues were collected and analysed using molecular biology techniques.

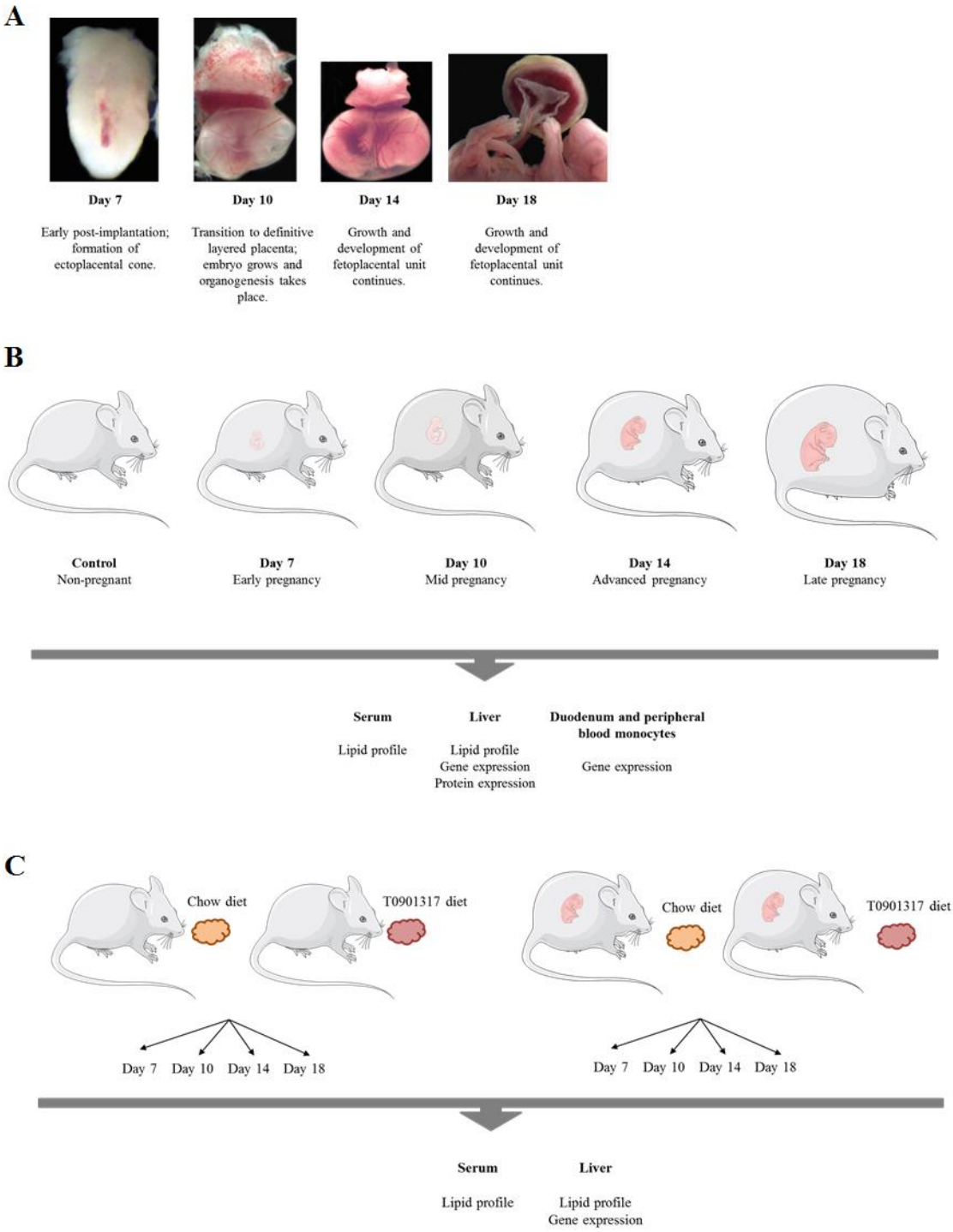


FIGURE 3.1: Schematic summary of the methodological approaches to study the contribution of LXR to the adaptations in maternal lipid metabolism during pregnancy. **A.** Development of mouse fetoplacental unit on days 7, 10, 14 and 18 of pregnancy. Images are adapted from (Croy et al., 2014). **B**. Schematic summary of an *in vivo* experiment aiming to examine the adaptations in lipid metabolic pathways during mouse pregnancy. **C**. Schematic summary of an *in vivo* experiment aiming to examine the impact of pharmacological LXR activation during pregnancy on the gestational adaptations in lipid metabolism.

3.3 Results

3.3.1 Alterations in hepatic and serum lipids in mouse pregnancy

In order to examine the impact of pregnancy on murine lipid metabolism, hepatic and serum lipids were profiled in mice at different stages of pregnancy: day 7 pc, corresponding to early pre-placentation pregnancy; day 10pc, representing early post-placentation pregnancy; day 14pc, corresponding to advanced pregnancy with established placentation and day 18pc representing late pregnancy. Virgin female mice were used as non-pregnant controls.

Initial characterisation revealed that relative liver-to-body weight ratio was significantly raised on day 7pc and was maintained unchanged until day 18pc (Figure 3.1A). Hepatic cholesterol content was also increased on days 7 and 10 of pregnancy ($p \leq 0.05$) (Figure 3.2B). There were no significant changes in the hepatic concentrations of triglycerides and free fatty acids in pregnant mice.

The liver plays a key role in the regulation of lipoprotein metabolism and modulates the distribution of circulating lipids in lipoprotein particles (Rui, 2014). Therefore, the temporal changes in serum lipid profiles in pregnant mice were examined. Our data suggest that total cholesterol and HDL-cholesterol levels decreased progressively from day 7pc onwards (Figure 3.2C) whereas the concentrations of LDL-cholesterol were reduced on day 10 of pregnancy and then returned to non-pregnant levels on day 18pc ($p \leq 0.05$). In contrast, serum triglyceride concentrations gradually increased starting from day 10 of mouse pregnancy onwards ($p \leq 0.05$). Serum levels of free fatty acids were not significantly altered during pregnancy.

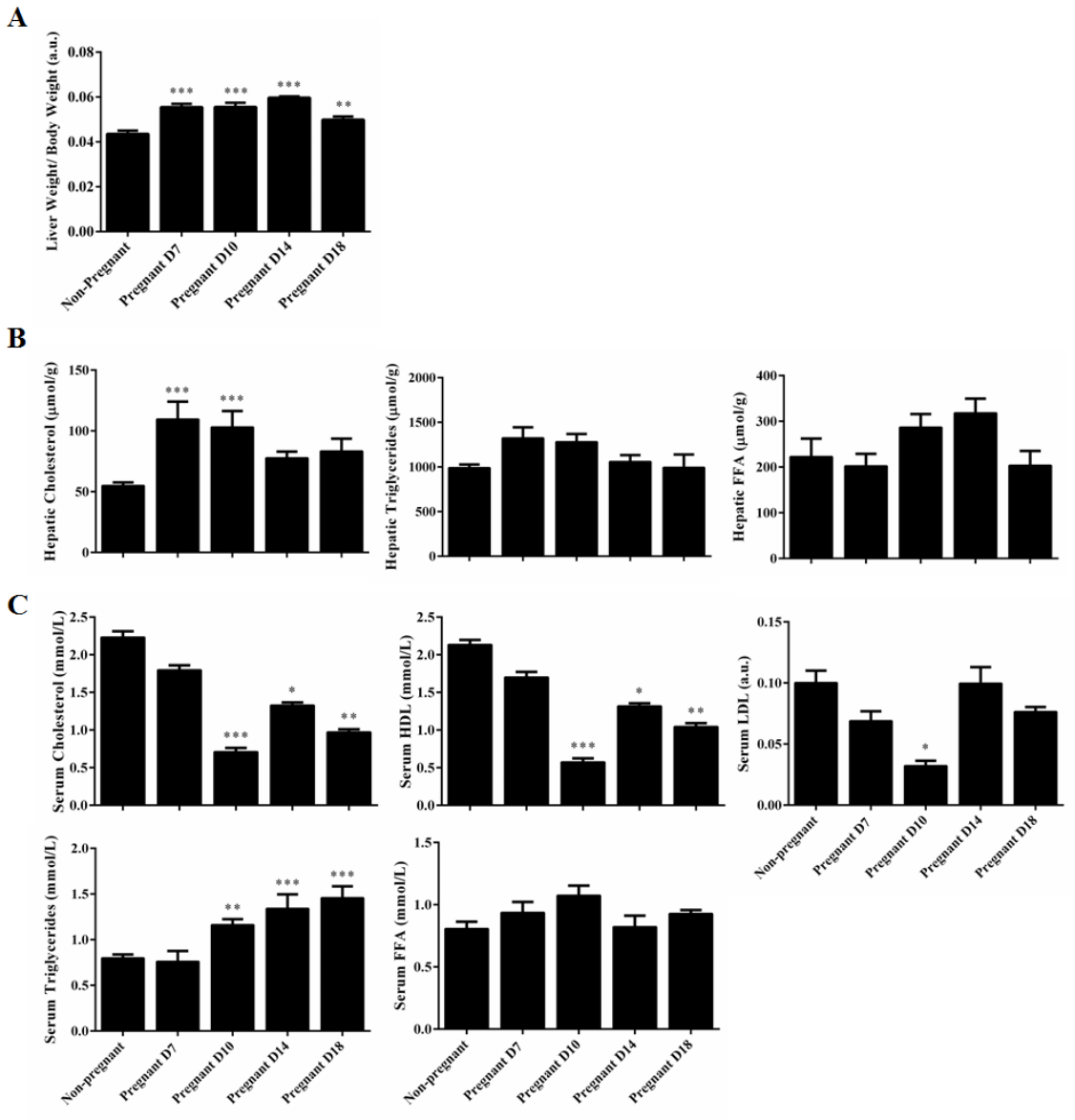


FIGURE 3.2: Impact of pregnancy on liver weight and biochemical lipid profiles. Wild-type mice were sacrificed on days 7, 10, 14 and 18 of pregnancy. Non-pregnant mice were used as controls. **A**. Relative liver weight (normalised to body weight). **B**. Total cholesterol, triglycerides and free fatty acids measured in mouse hepatic lipid extracts. **C**. Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and free fatty acids measured in mouse serum. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of pregnant versus non-pregnant group. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.2 Alterations in hepatic LXR transcriptome during mouse pregnancy

LXR plays a pivotal role in the regulation of whole-body lipid metabolism by regulating *de novo* lipogenesis as well as cholesterol uptake and clearance in the liver. To determine whether the LXR transcriptome changes during mouse pregnancy and thereby contributes to the gestational alterations in lipid metabolism, the expression of LXR target genes was quantified in the livers of mice sacrificed on days 7, 10, 14 and 18pc. Virgin female mice were used as non-pregnant controls.

First, we studied the impact of pregnancy on the expression of LXR targets involved in hepatic *de novo* lipogenesis (Figure 3.3A). The expression of Acc1 and Scd1 was raised on day 7 of pregnancy and then it was reduced below non-pregnant levels on days 10, 14 and 18pc ($p \leq 0.05$). In contrast, Fas was significantly upregulated on day 7pc but then it returned to non-pregnant levels. The expression of Srebp1c, on the other hand, appeared to be consistently downregulated throughout mouse pregnancy ($p \leq 0.05$). The mRNA levels of Cyp51a1 were significantly reduced from day 10 of pregnancy onwards.

Moreover, we examined how the transcript levels of hepatic transporters mediating the uptake of cholesterol from plasma lipoproteins change throughout mouse pregnancy (Figure 3.3B). Our results showed that the expression of the Ldlr was reduced from day 10pc onwards whereas the mRNA levels of Srb1 was raised on day 7pc and then reduced on gestational days 14 and 18.

Finally, we investigated the impact of pregnancy on biliary cholesterol excretion and bile acid synthesis (Figure 3.3C). Based on our data, the expression of Abcg5 was decreasing progressively throughout gestation ($p \leq 0.05$). There was a minimal increase in the transcript abundance of Abcg8 on day 7pc ($p \leq 0.05$) whereas the mRNA levels of this transporter were significantly reduced from day 10 of pregnancy onwards. On the other hand, Cyp7a1 expression was decreased on gestational days 14 and 18 ($p \leq 0.05$).

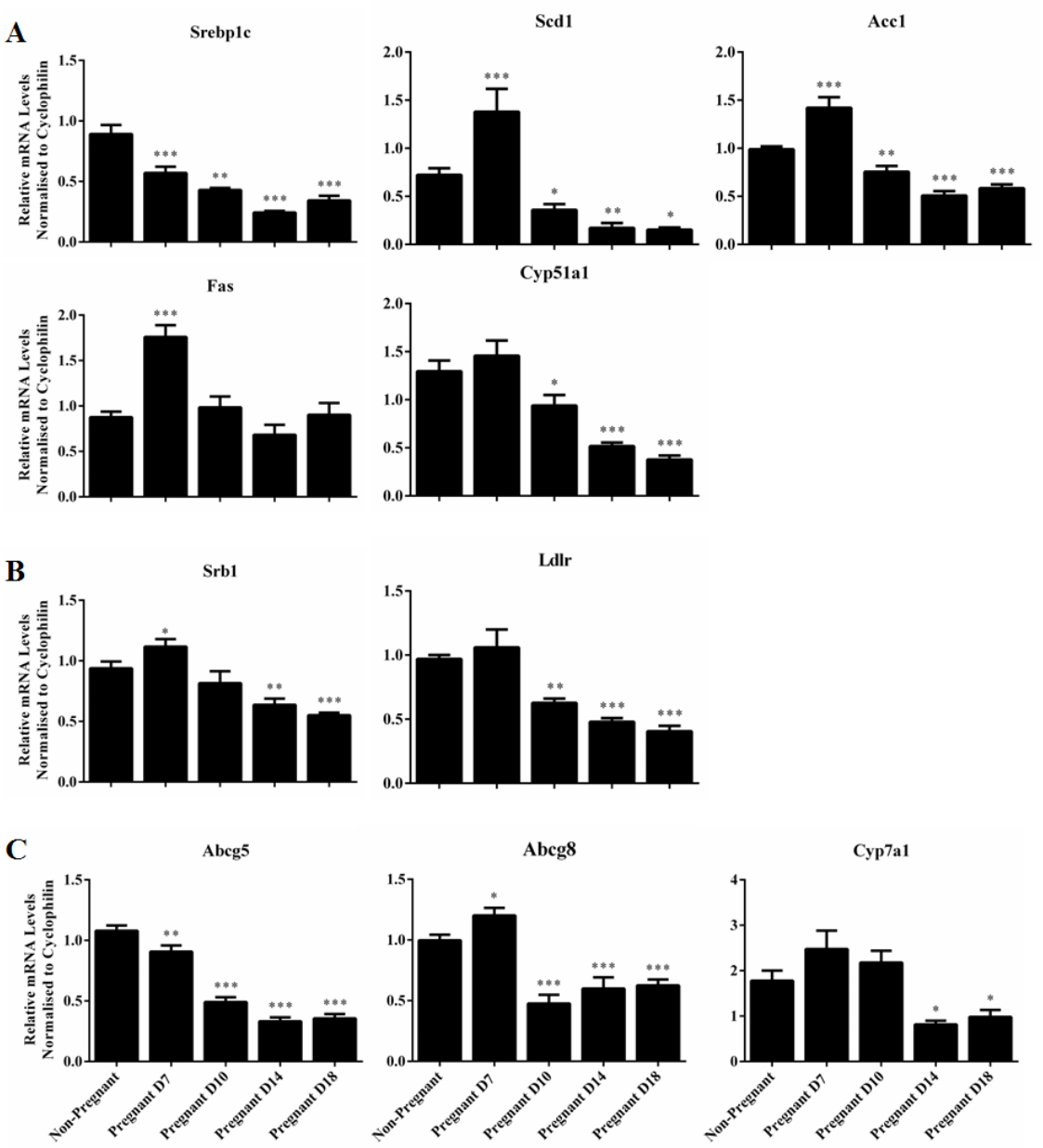


FIGURE 3.3: Expression of LXR target genes in mouse liver during pregnancy. Wild-type mice were sacrificed on days 7, 10, 14 and 18 of pregnancy. Non-pregnant mice were used as controls. Relative mRNA levels were quantified using RT-PCR. **A**. Enzymes involved in *de novo* lipogenesis (Srebp1c, Fas, Scd1, Acc1 and Cyp51a1). **B**. Cholesterol uptake receptors (Ldlr and Srb1).**C**. Cholesterol transporters (Abcg5 and Abcg8) and bile acid biosynthetic enzyme (Cyp7a1). Results are represented as mean ± SEM (n=6-8). *p≤0.05, **p≤ 0.01, ***p ≤ 0.001, comparison of pregnant versus non-pregnant group. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.3 Impact of pregnancy on the expression of LXR in the liver

Since the transcript abundance of *bona fide* LXR target genes involved in hepatic *de novo* lipogenesis as well as hepatic cholesterol uptake and clearance were altered during mouse pregnancy, we examined the impact of gestation on the expression of the two LXR isoforms in rodent liver.

LXRα is the dominant isoform of this nuclear receptor in the liver (Alberti et al., 2001). Its mRNA and protein levels were unchanged at the gestational time-points tested (Figure 3.4A and B). The transcript abundance of Lxrβ was raised on days 7 and 10pc, and was reduced on day 18pc when compared to non-pregnant levels ($p \leq 0.05$) (Figure 3.4A); the protein expression of this nuclear receptor isoform did not appear to change substantially during mouse pregnancy (Figure 3.3B).

LXR forms an obligate heretodimer with RXR in order to drive the expression of its target genes (Janowski et al., 1996) and therefore we examined whether the expression of this transcription factor changes in response to pregnancy, thus affecting the transcript abundance of key cholesterol homeostatic genes. Temporal mRNA profiling revealed that Rxrα levels were significantly upregulated on day 7pc and then significantly reduced on day 14pc as compared to non-pregnant levels (Figure 3.4A)

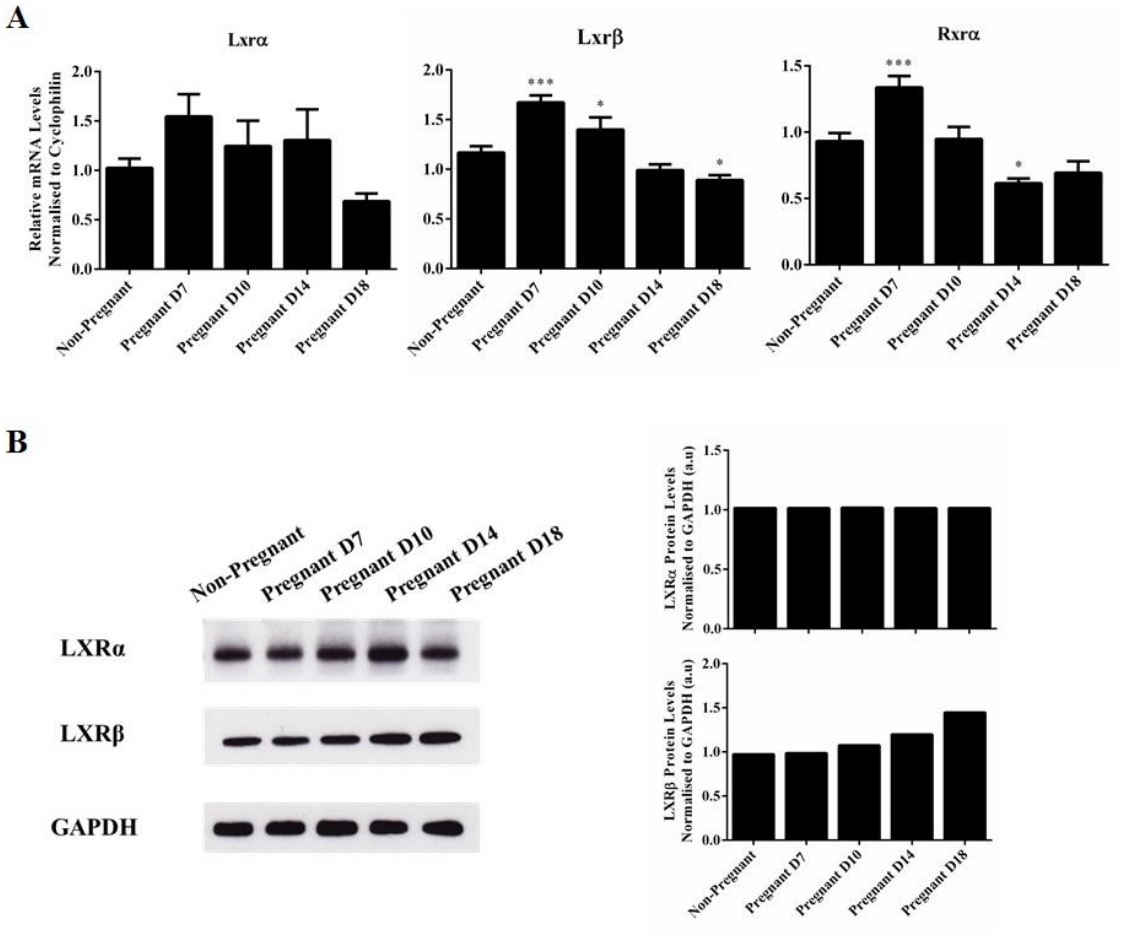


FIGURE 3.4: Expression of LXR and RXR in mouse liver during pregnancy. Wild-type animals were sacrificed on days 7, 10, 14 and 18 of pregnancy. Non-pregnant animals were used as controls. **A.** Gene expression of Lxrα, Lxrβ and Rxrα. Results are represented as mean ± SEM (n=6-8). *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, pregnant versus non-pregnant group. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing. **B.** Protein expression of LXRα and LXRβ. Western blot analysis was employed to determine LXRα and LXRβ protein levels in pooled whole-cell liver lysates (n=6-8). GAPDH was tested as a loading control. Densitometry analysis of protein bands was performed with the ImageJ software.

3.3.4 Impact of pregnancy on the expression of LXR co-factors in the liver

In the absence of a ligand, LXR is bound to the promoter region of its target genes in association with co-repressors and thereby blocks transcription (Chen and Evans, 1995). Upon ligand-activation, LXR is bound by co-activators which facilitate the assembly of transcriptional machinery and drive the mRNA expression of *bona fide* targets (Son and Lee, 2010). Therefore, we examined whether the alterations in the transcript abundance of LXR target genes could stem from changes in the distribution of co-factors in mouse liver during pregnancy.

mRNA profiling of known LXR co-factors revealed that the expression of Pgc1α was reduced in all pregnant groups while the transcript levels of Pgc1β and Scr1 were downregulated from day 10pc onwards ($p \leq 0.05$) (Figure 3.5A). Moreover, the expression of the published PGC1 target genes Medium-chain acyl-CoA dehydrogenase (Mcad) and Cytochrome C (Liu and Lin, 2011)were significantly decreased at all gestational time-points as compared to non-pregnant controls (Figure 3.4B).

Hepatic expression of LXR co-repressors was also studied (Figure 3.5C). The mRNA levels of Ncor were significantly reduced from day 10 of pregnancy onwards. In contrast, the transcript abundance of Smrt was raised on day 7pc and then was downregulated at all other stages of pregnancy as compared to non-pregnant controls ($p \leq 0.05$). No significant changes were detected in the expression of Rip140.

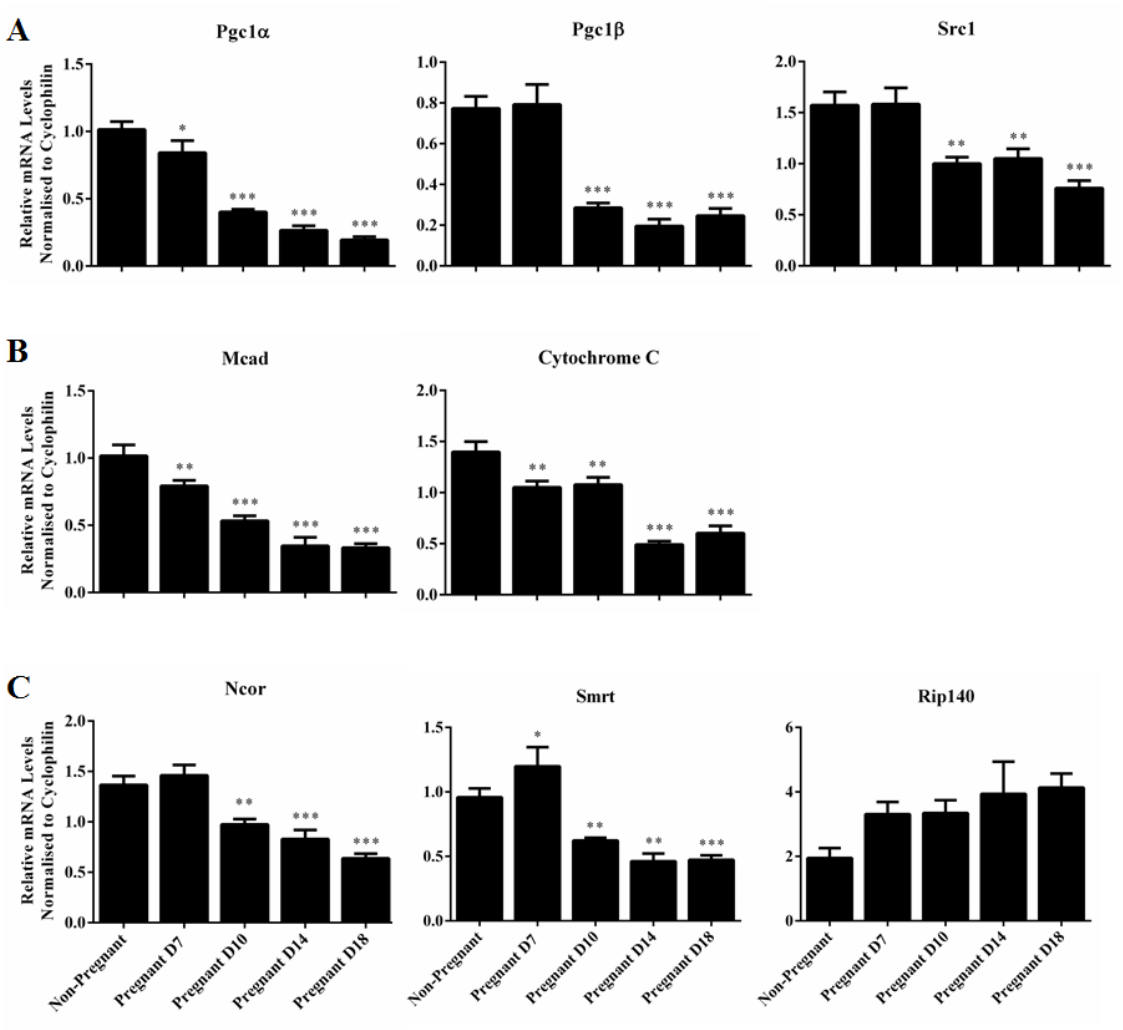


FIGURE 3.5: Expression of LXR co-factors in the liver during pregnancy. Wild-type mice were sacrificed on days 7, 10, 14 and 18 of pregnancy. Non-pregnant mice were used as controls. Relative mRNA levels were quantified using RT-PCR. **A**. LXR co-activators. **B**. PGC1 target genes. **C**. LXR co-repressors. Results are represented as mean ± SEM (n=6-8). *p≤0.05, **p≤ 0.01, ***p ≤ 0.001, comparison of pregnant versus non-pregnant group. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.5 Alterations in LXR transcriptome in extrahepatic tissues during mouse pregnancy

LXR controls whole body lipid metabolism not only by regulating *de novo* lipogenesis and cholesterol uptake and clearance in the liver but also by modulating sterol absorption in the intestine as well as efflux of cholesterol from extrahepatic tissues (Calkin and Tontonoz, 2012). Consequently, the expression of LXR target genes was profiled in the duodenum of pregnant mice on days 7, 10, 14 and 18pc and also in murine blood monocytes on days 7 and 14 of pregnancy. Virgin female mice were used as non-pregnant controls.

First, we studied the impact of pregnancy on key intestinal transporters mediating the absorption of luminal cholesterol. The mRNA levels of both Lxr isoforms and their target genes Abca1, Abcg5, Abcg8 and Npc1l1 did not change in response to pregnancy in mouse duodenum (Figure 3.6A).

Moreover, we tested if there were any pregnancy-induced changes in cholesterol homeostatic pathways, controlled by LXR, in peripheral blood monocyte cells (PBMCs). The transcript abundance of Arl7, Abcg1, Ldlr, Fas, Scd1 and Acc1 was not altered at any of the gestational time-points (Figure 3.6B). In contrast, the mRNA levels of Lxrα and Lxrβ were significantly raised on day 14 of pregnancy as compared to non-pregnant controls.

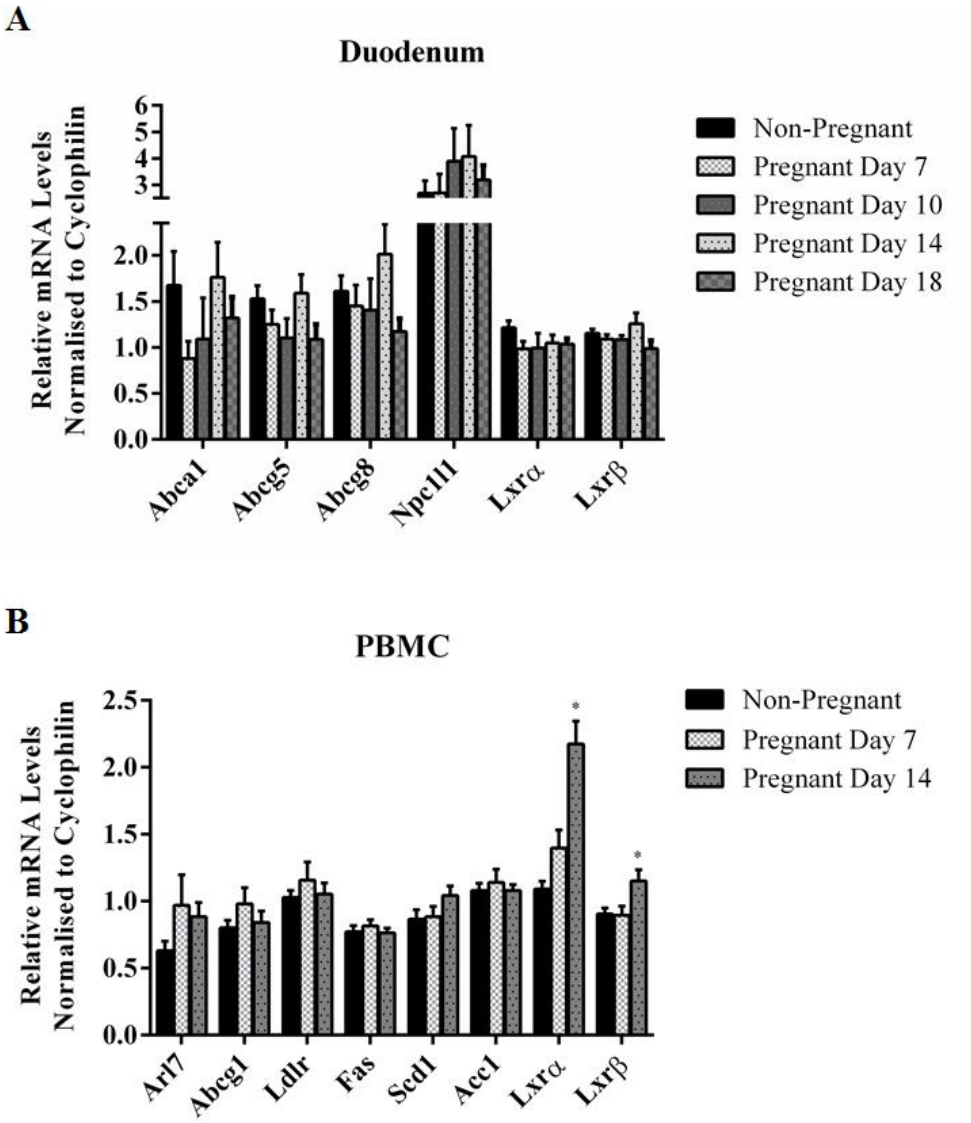


FIGURE 3.6: Expression of LXR and its target genes in extrahepatic tissues. Relative mRNA levels were quantified using RT-PCR. **A**. Gene expression of Lxr and its target genes in duodenum. Wild-type mice were sacrificed on days 7, 10, 14 and 18 of pregnancy. Non-pregnant mice were used as controls. **B**. Gene expression of Lxr and its target genes in peripheral blood monocyte cells (PBMC). Wild-type mice were sacrificed on days 7 and 14 of pregnancy. Non-pregnant mice were used as controls. Results are represented as mean ± SEM (n=6-8). *$p \leq 0.05$, comparison of pregnant versus non-pregnant group. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.6 Impact of pharmacological activation of LXR on physiological adaptations of pregnancy

In order to investigate the role of LXR in the gestational alterations in lipid homeostasis, we examined whether pharmacological activation of LXR during pregnancy affects lipid metabolic adaptations. To address this question, pregnant mice were fed RM3 standard chow diet (chow) or T0901317 diet for 7, 10, 14 and 18 days following the identification of the plug. Non-pregnant female mice fed a T0901317 or chow diet for the same number of days were used as controls.

First, we examined the effect of the T0901317 feeding on total body weight. Our results indicated that T0901317-fed pregnant mice had significantly lower total body weight from day 12 onwards when compared to control pregnant mice (Figure 3.7A).

Since LXR plays a key role in the regulation of hepatic lipid metabolism, we studied the effect of pharmacological activation of LXR during mouse pregnancy on the changes in liver size described in section 3.2.1. We found that T0901317 feeding resulted in an increase in the liver-to-body weight ratio, however, the magnitude was higher in T0901317-fed pregnant animals than in controls matched for gestational age or diet ($p \leq 0.05$) (Figure 3.7B).

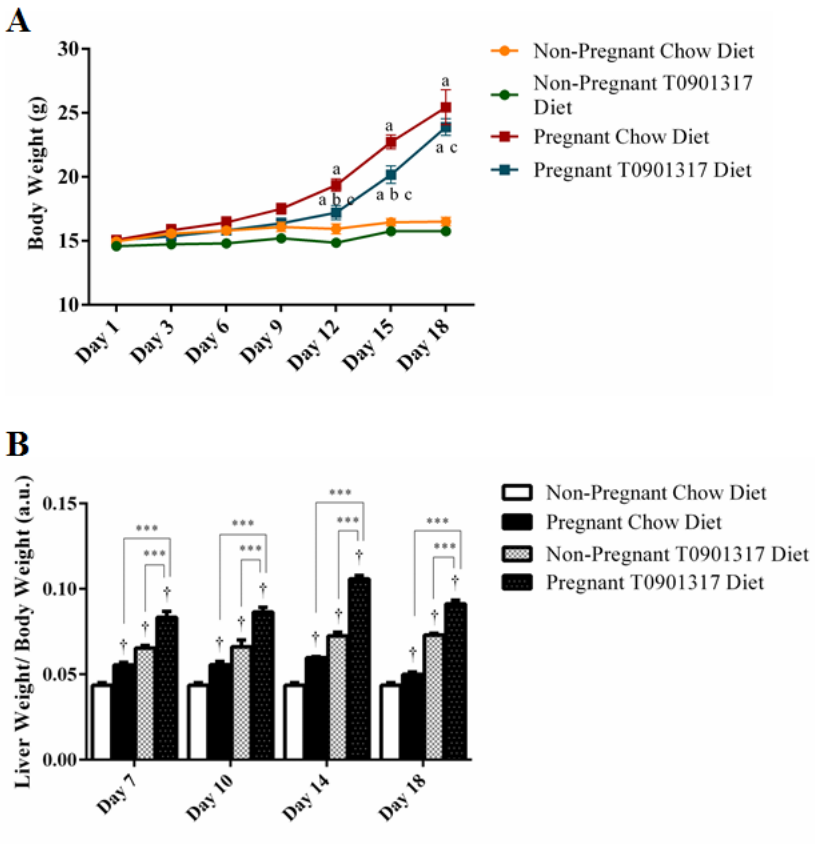


FIGURE 3.7: Impact of pharmacological activation of LXR during mouse pregnancy on body weight and relative liver weight. Pregnant/non-pregnant wild-type mice were fed T0901317 diet/chow diet for 7, 10, 14 and 18 days and then were sacrificed. Feeding of pregnant mice with T0901317 diet was initiated upon the identification of a vaginal plug. Non-pregnant animals fed with standard chow were represented as day 0 of pregnancy. **A**. Changes in body weight. Animals were weighed every 3 days for a period of 18 days. Results are represented as mean ± SEM (n=5). a ≤0.05 comparison of day 3-18 groups versus day 1 group, b ≤0.05 comparison of T0901317-fed pregnant group versus diet-matched non-pregnant group, c ≤0.05 T0901317-fed pregnant group versus matched chow-fed pregnant group. **B**. Liver-to-body weight ratios. Results are represented as mean ± SEM (n=5-8). † p≤0.05 comparison of non-pregnant T0901317 diet/ pregnant chow diet/ pregnant T0901317 diet group versus non-pregnant chow diet group, *p≤0.05, **p≤ 0.01, ***p ≤ 0.001, comparison of indicated groups. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.7 Impact of pharmacological activation of LXR on lipid biochemical profile during pregnancy

As shown in 3.2.6, mouse lipid profiles change during pregnancy. Since we have hypothesised that alteration in the activity of LXR could contribute to these alterations, we examined whether pharmacological activation of LXR could reverse the gestation-induced alterations in lipid metabolism. For this purpose, we tested the impact of T0901317-feeding throughout murine pregnancy on hepatic and lipid biochemistry.

As expected, non-pregnant mice fed with T0901317 diet presented with decreased hepatic cholesterol content as well as raised hepatic triglycerides and free fatty acids levels ($p \leq 0.05$) (Figure 3.8A). The same mice also had significantly raised levels of serum total cholesterol and HDL-cholesterol; no changes were detected in the serum concentrations of LDL-cholesterol, triglycerides and free fatty acids (Figure 3.8B).

Pharmacological activation of LXR during mouse pregnancy significantly reduces gestation-induced cholesterol accrual in the liver (Figure 3.8A). Pregnant and non-pregnant mice challenged with a T0901317 feeding had raised levels of hepatic triglycerides and free fatty acids at all gestational time-points as compared to chow-fed pregnant animals ($p \leq 0.05$). In the serum, the concentrations of total cholesterol, HDL- and LDL-cholesterol were decreased during normal mouse pregnancy; however, administration of T0901317 abrogated this reduction ($p \leq 0.05$) (Figure 3.8B). Also, as opposed to pregnant mice fed a standard diet, where serum triglycerides and free fatty acid levels increased as gestation progressed, in T0901317-fed pregnant mice, triglyceride and free fatty acid abundance remained unaltered throughout pregnancy and their levels were significantly lower than the ones measured in normal pregnancy.

On the other hand, a comparison of pregnant versus non-pregnant mice fed with the T0901317 diet revealed that the impact of the LXR agonist on hepatic cholesterol levels and serum concentrations of total and HDL-cholesterol was less marked in pregnant animals than in the non-pregnant ones.

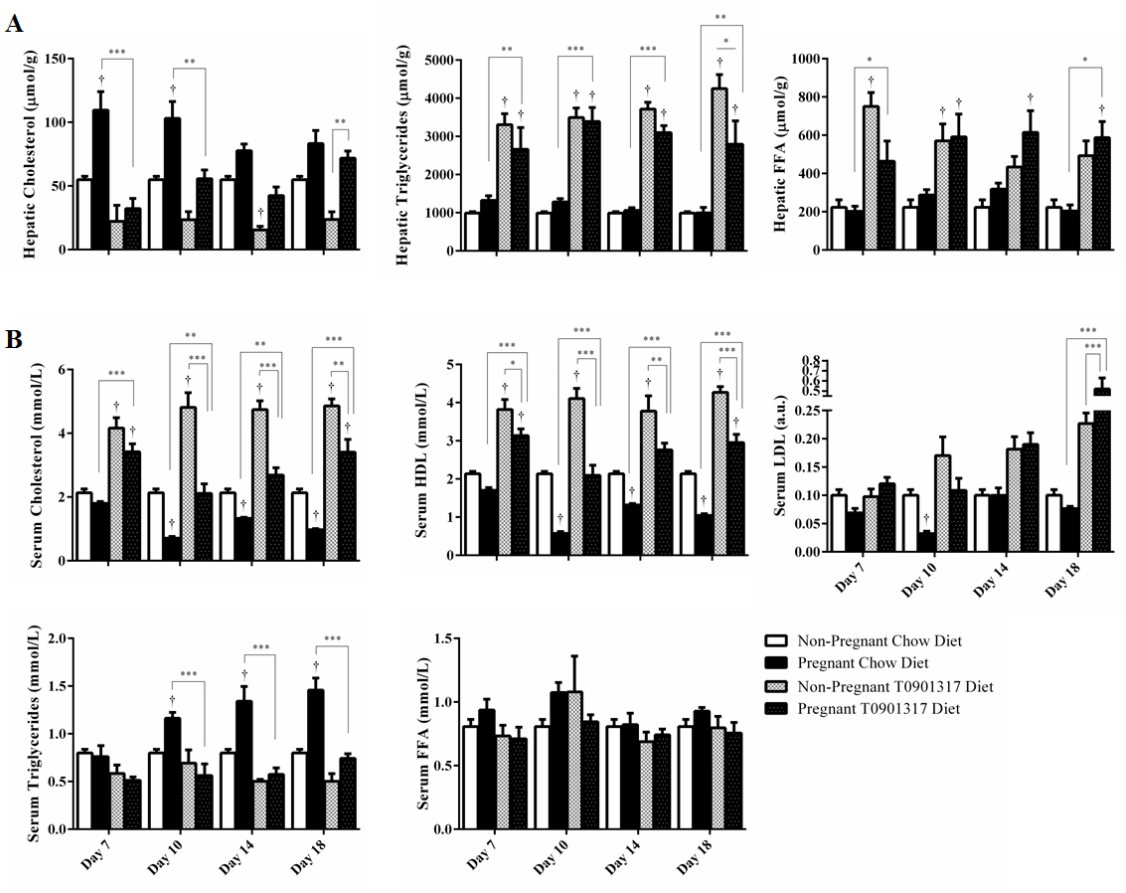


FIGURE 3.8: Impact of pharmacological activation of LXR during mouse pregnancy hepatic and serum lipid profiles. Pregnant/non-pregnant wild-type mice were fed T0901317 diet/chow diet for 7, 10, 14 and 18 days and then were sacrificed. Feeding of pregnant mice with T0901317 diet was initiated upon the identification of a vaginal plug. Non-pregnant animals fed with standard chow were represented as day 0 of pregnancy. **A**. Total cholesterol, triglycerides and free fatty acids measured in mouse hepatic lipid extracts. **B**. Total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and free fatty acids measured in mouse serum. Results are represented as mean ± SEM (n=5-8). † $p \leq 0.05$ comparison of non-pregnant T0901317 diet/ pregnant chow diet/ pregnant T0901317 diet group versus non-pregnant chow diet group, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, comparison of indicated groups. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

3.3.8 Impact of LXR activation during pregnancy on the hepatic LXR transcriptome

To determine whether LXR is functional during mouse gestation and whether the decrease in the mRNA levels of key metabolic genes in mouse liver post-placentation is LXR-dependent, this nuclear receptor was pharmacologically activated throughout mouse pregnancy; pregnant mice were challenged with a T0901317 diet for this purpose.

As expected, feeding of non-pregnant mice with the T0901317-supplemented diet significantly increased the expression of LXR target genes involved in fatty acid biosynthesis (Srebp1c, Acc1, Scd1) (Figures 3.9A), hepatic cholesterol uptake (Srb1 and Ldlr) and biliary cholesterol clearance (Abcg5, Abcg8, Cyp7a1) (Figure 3.9A). Cyp51a1 is negatively regulated by LXR activation (Wang et al., 2008) and therefore its mRNA expression was significantly decreased in non-pregnant mice challenged with the agonist-supplemented diet (Figures 3.9A).

On gestational day 7, the T0901317-enhanced expression of Acc1, Scd1, Abcg8, Cyp7a1, Ldlr, and Srb1 was further increased in the livers of mice challenged with the agonist-supplemented diet during pregnancy ($p \leq 0.05$).

From day 10 of pregnancy onwards, there was a shift to decreased mRNA levels of the vast majority of LXR target genes and the same pattern was observed in the T0901317-fed mice (e.g. Srebp1c, Scd1, Acc1, Ldlr, Srb1, Abcg5, Abcg8 and Cyp7a1), though the latter maintained expression levels higher than the ones detected in normal pregnancy ($p \leq 0.05$).

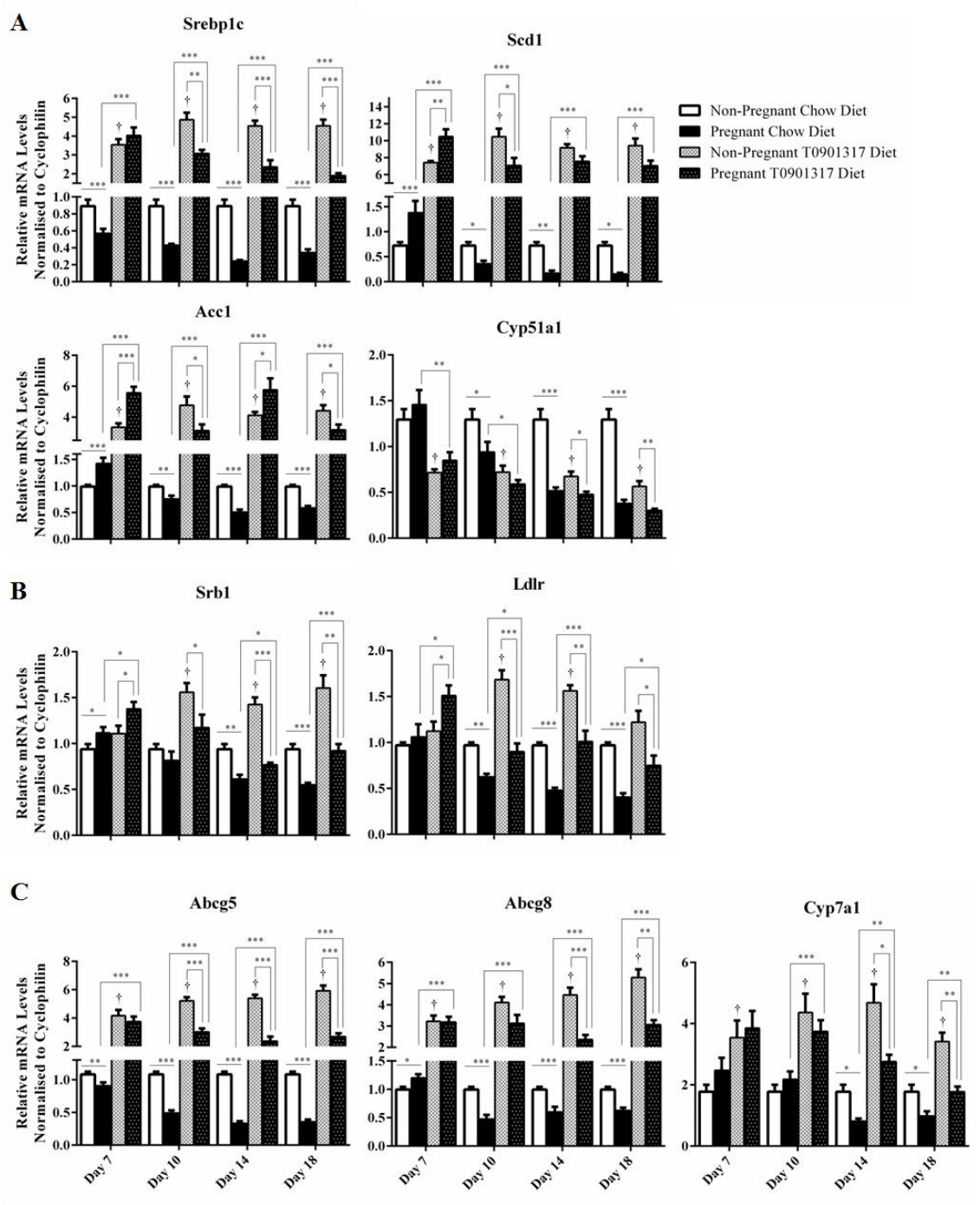


FIGURE 3.9: Impact of pharmacological activation of LXR during mouse pregnancy on cholesterol homeostatic pathways in the liver. Pregnant/non-pregnant wild-type mice were fed T0901317 diet/chow diet for 7, 10, 14 and 18 days and then were sacrificed. Feeding of pregnant mice with T0901317 diet was initiated upon the identification of a vaginal plug. Non-pregnant animals fed with standard chow were represented as day 0 of pregnancy. **A**. Enzymes involved in *de novo* lipogenesis (Srebp1c, Fas, Scd1, Acc1 and Cyp51a1). **B**. Cholesterol uptake receptors (Ldlr and Sreb1). **C**. Cholesterol biliary transporters (Abcg5 and Abcg8) and bile acid biosynthetic enzyme (Cyp7a1). Results are represented as mean ± SEM (n=5-8). † $p \leq 0.05$ comparison of non-pregnant T0901317 diet/ pregnant chow diet/ pregnant T0901317 diet group versus non-pregnant chow diet group, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, comparison of

indicated groups. Data were analysed using multiple measures of ANOVA with Newman-Keuls post-hoc testing.

In conclusion, the data described in this chapter demonstrate that mouse gestational adaptations in lipid homeostasis include raised hepatic cholesterol content, decreased levels of circulating cholesterol and elevated serum triglycerides. Moreover, LXR signalling contributes to the enhanced lipogenesis in early mouse pregnancy by increasing fatty acid biosynthesis and cholesterol uptake in the liver. Following mouse placenta formation (day 10 of mouse pregnancy), there is a gradual downregulation of LXR targets involved in hepatic lipogenesis, cholesterol uptake and clearance (summarised in Figure 3.10). However, pharmacological activation of LXR not only blunts the reduction of these genes but also reverses the changes in hepatic and serum lipid profiles observed during normal murine pregnancy.

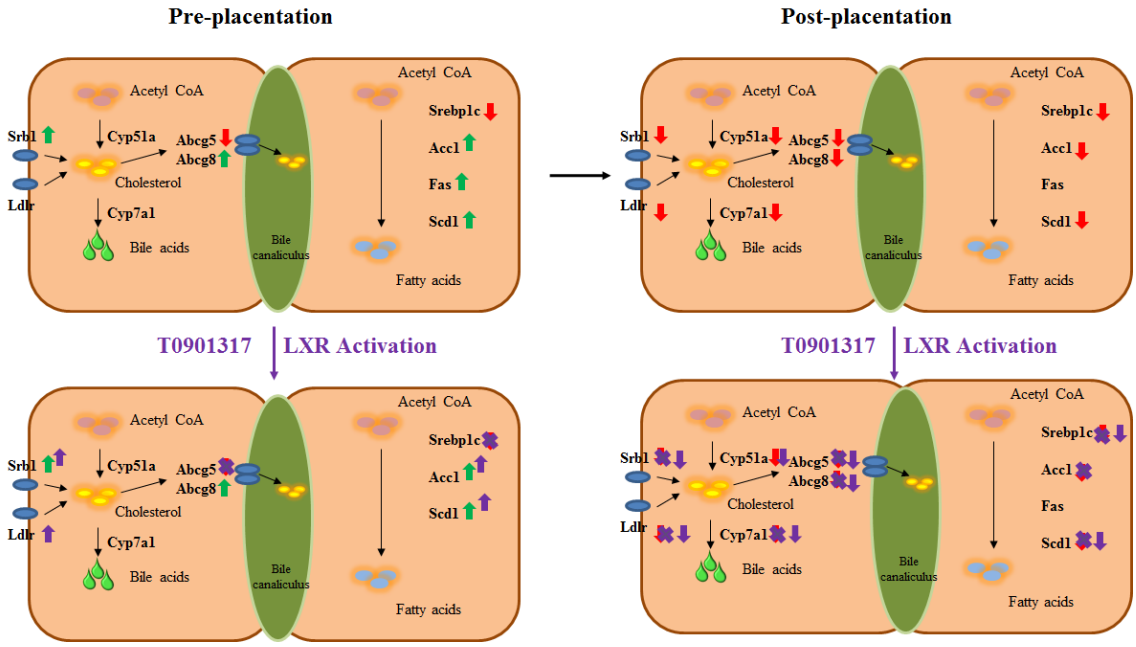


FIGURE 3.10: Pharmacological activation of LXR during murine pregnancy reverses the gestational adaptations in the expression of lipid metabolic genes in the liver. LXR target genes are differentially regulated during mouse pregnancy. Pre-placentation, there is increased fatty acid biosynthesis (Acc1, Fas, Scd1 are increased) and cholesterol uptake (Srb1 is upregulated). Post-placentation, there is reduced cholesterol uptake (Srb1 and Ldlr are downregulated), *de novo* lipogenesis (Cyp51a1, Srebp1c, Acc1 and Scd1 are decreased) and cholesterol clearance (Abcg5, Abcg8 and Cyp7a1). Pharmacological activation of T0901317 during pregnancy increased the expression of LXR targets genes above their normal pregnancy levels. Despite the presence of T0901317, LXR is further activated in early pregnancy thus promoting increased cholesterol uptake and fatty acid biosynthesis. T0901317 is able to restore LXR activation post-placentation; however, pregnancy is still associated with reduced *de novo* lipogenesis, cholesterol uptake and clearance.

3.4 Discussion

3.4.1 Hepatic LXR signalling is altered during pregnancy and this is an essential adaptation to facilitate altered maternal lipid homeostasis

Physiological pregnancy in humans is associated with the development of hyperlipidaemia which encompasses increases in plasma triglyceride and cholesterol levels during advanced-to-late gestation (Alvarez et al., 1996). Although the molecular mechanisms contributing to gestational hypertriglyceridemia have been extensively studied, there is a substantial gap in our knowledge regarding the adaptations in the lipid metabolism of the mother which potentiate the rise in plasma total and lipoprotein-cholesterol levels during pregnancy. Therefore, the aim of this study was to investigate the contribution of LXR, a master regulator of cholesterol homeostasis, to the changes in cholesterol metabolism during pregnancy (Kalaany and Mangelsdorf, 2006, Calkin and Tontonoz, 2012).

To examine how cholesterol homeostasis adapts in the course of murine pregnancy, we profiled the changes in serum and hepatic lipid biochemistry at different stages of mouse gestation. Our results indicated that, unlike in humans, total serum cholesterol decreases during mouse pregnancy and this pattern was reflected by the changes in the abundance of the sterol in the HDL lipoprotein fraction. In contrast, the content of cholesterol in LDL particles was declining until day 10 and then it returned to pre-pregnancy levels. At the same time it was observed that the hepatic cholesterol load was maximal during early mouse pregnancy and then gradually decreased from day 10 onwards. Since the physiological role of LDL particles is to transfer cholesterol from the liver to peripheral tissues such as the placenta, it is conceivable that liver functions as a repository for the sterol, accumulating it during early pregnancy, and then releasing it as gestation advances in order to satisfy the metabolic demands of the developing fetus.

Our data showed that serum triglyceride concentrations increased throughout murine pregnancy while the levels of free fatty acids in the serum remained relatively constant. Conversely, mouse pregnancy appeared to have a limited effect on the concentrations of free fatty acids and triglyceride in the liver, which were minimally raised at different stages of gestation.

LXR plays an encompassing role in the regulation of all domains of cholesterol metabolism in the body, namely control of *de novo* lipogenesis and biliary excretion of the sterol in the liver, primary and secondary absorption of the sterol in the intestine as well as the influx and the efflux of sterols in peripheral tissues (Kalaany and Mangelsdorf, 2006, Calkin and Tontonoz, 2012). The expression of LXR target genes in the duodenum was not changed during mouse pregnancy suggesting that the gestational adaptations in cholesterol homeostasis in the mouse

are unlikely to result from changes in the capacity of the intestine to utilise dietary and biliary sterols. Moreover, the LXR transcriptome in peripheral blood monocytes also remained unaltered during mouse pregnancy indicating that changes in the uptake and clearance of sterols in peripheral tissues are unlikely to contribute to the alterations in cholesterol abundance in murine gestation. Due to the fact that the concentrations of serum cholesterol change in a contrasting manner in humans and mice, we were not able to examine the molecular mechanisms which could allow maternal tissues to limit the internalisation of the exogenous sterols which circulate abundantly throughout human gestation. In order to circumvent this limitation of our experimental model, mice will be fed with a cholesterol-enriched diet during pregnancy in order to recapitulate human gestational hypercholesterolemia. Moreover, peripheral blood monocytes from late pregnant women and non-pregnant control women (who are in the follicular phase of their phase of their menstrual cycle; this criterion is key in order to minimise the impact of reproductive hormones on nuclear receptor signalling) will be isolated and analysed in order to determine whether the activity of extrahepatic LXR is modified during non-complicated human pregnancy.

Our results demonstrated that the expression of the LXR transcriptome in the liver changes during mouse pregnancy and this could potentially contribute to the gestational adaptations in cholesterol homeostasis in the mouse. Specifically, the transcript abundance of the key fatty acid biosynthetic genes Fas, Scd1 and Acc1 was upregulated during early mouse pregnancy thereby facilitating the enhanced lipogenesis during this period. Interestingly, in advanced-to-late murine pregnancy, the expression of LXR target genes involved in *de novo* lipogenesis as well as hepatic cholesterol uptake and clearance decreased progressively in parallel with the reduction in cholesterol levels in the liver. This switch is possibly an adaptation which could limit the loss of cholesterol during pregnancy. Moreover, the observed changes in the expression of lipid metabolic genes in the liver strongly suggested that the activity of LXR could be altered during pregnancy. However, the protein levels of both LXRα and LXRβ remained relatively constant during mouse pregnancy indicating that other mechanisms contribute to the attenuation of LXR activity in the liver.

Nuclear receptors control the expression of their target genes by recruiting transcriptional co-activators and co-repressors in response to ligand-dependent activation. Therefore, we examined whether pregnancy could alter the activity of LXR by modulating the availability of its co-factors. Our results demonstrated for the first time that the mRNA expression of the transcriptional co-activators Pgc1α and Pgc1β was severely diminished during pregnancy and this reduction could have an impact on hepatic physiology since the transcript abundance of the canonical PGC-1 targets Mcad and Cytochrome C was reduced in parallel. PGC1α and PGC1β are multifunctional transcriptional regulators that act as "molecular switches" in various metabolic pathways by interacting with different nuclear receptor and increasing their

transcriptional activity in a tissue-specific manner (Liu and Lin, 2011). Both PGC1 isoforms play significant roles in the control of energy and lipid metabolism by serving as a platform for the recruitment and assembly of various chromatin-remodelling and histone-modifying enzymes to alter the local chromatin architecture, making it transcription-permissive (Liu and Lin, 2011). Also, PGC1 could potently activate gene expression by displacing transcriptional repressor complexes (Liu and Lin, 2011). Recent studies have demonstrated that in the liver PGC1α orchestrates metabolic changes in glucose and lipid metabolism as well as bile acid homeostasis (Liu and Lin, 2011). PGC1β, on the other hand, promotes hepatic lipogenesis and VLDL secretion by co-activating LXR and SREBP1 (Lin et al., 2005). Therefore, it is possible that the attenuation in LXR activity in the livers of pregnant mice could result from the decreased abundance of PGC1 co-activators during this period. However, protein quantification studies are need to test whether the changes in the mRNA levels of these transcriptional regulators reflect actual modifications in their protein abundance in pregnant mice. Most importantly, chromatin immunoprecipitation experiments are needed to determine whether pregnancy affects the binding of LXR itself or PGC1α/β to the promoter sequences of cholesterol homeostatic genes in the liver.

Another possible explanation for attenuation of LXR signalling is that oxysterol homeostasis could also change during pregnancy thereby reducing the transcriptional activation of this nuclear receptor. Oxysterols are cholesterol derivatives which function as endogenous ligands of LXR, activating the expression of its target genes both *in vivo* and *in vitro* (Lehmann et al., 1997). At present, there are no published reports on the impact of pregnancy on the abundance of these molecules. Since oxysterols result from the oxygenation of cholesterol, it is conceivable that the reduction in the levels of this sterol in the sera of pregnant mice is likely to result in diminished availability of all of its derivatives and subsequent downregulation of LXR signalling. Therefore, measurement of oxysterol enrichment during murine pregnancy is an essential step that should allow us to better understand the physiological pathways that regulate lipid metabolism throughout gestation. Furthermore, oxysterols can be enzymatically reduced by sulphotransferases and the resulting sulphated oxysterol metabolites (e.g. 5-cholesten-3β, 25-diol 3-sulfate) are able to inhibit LXR signalling in the liver (Chen et al., 2007). Hydroxysteroid sulphotransferases are not expressed in the liver, but they are synthesised in other tissues, such as placenta (Shimizu et al., 2003), thus allowing for extrahepatic metabolism of oxysterols (Chen et al., 2007, Geese and Raftogianis, 2001). Therefore, increased expression of sulphotransferases in mid-to-late pregnancy might cause an increase in the levels of sulphated oxysterols that can be taken up by the liver in order to be metabolised into bile acids. It is possible that, sulphated oxysterols follow the same metabolic pathways and thus return to the liver where they impair LXR signalling in mid-to-late pregnancy.

Aiming to determine whether changes in the activity of LXR mediate the gestational adaptations in lipid homeostasis, we challenged pregnant mice with the synthetic LXR agonist T0901317 (Schultz et al., 2000) through the diet. This agonist was selected based on the fact that it is able activate both LXR isoforms, has a marked potency (Schultz et al., 2000) and also accumulates to high concentrations in mouse liver, thus facilitating maximum activation of LXR in this organ (Zhang et al., 2012). The final dose of 30mg of T0901317 per kilogram of mouse weight per day was chosen following a comprehensive literature review which revealed that this compound was administered in doses varying between 3-50mg/kg/day depending on the duration of the treatment (e.g. 3mg/kg/day for 8 weeks (Terasaka et al., 2003) and 50mg/kg/day for 7 days (Schultz et al., 2000)). A key consideration during the design of the study was to deliver the agonist at a dose sufficient to maintain a constant level of LXR activation throughout pregnancy and to reverse the reduction in the expression of LXR targets observed from gestational day 10 onwards; on the other hand, the dose of T0901317 was low enough to avoid harmful effects on murine physiology such as reduced rates of embryo implantation, miscarriages and fetal death.

Pharmacological activation of LXR was able to reverse the gestational adaptations in lipid homeostasis in the mouse, increasing total, HDL and LDL-cholesterol levels during pregnancy, reducing the hepatic cholesterol content during early gestation and also decreasing the abundance of triglycerides in the sera of pregnant mice. Moreover, administration of T0901317 during pregnancy was sufficient to raise the transcript abundance of hepatic LXR targets and to maintain them at levels higher than the ones detected during normal pregnancy. These data collectively demonstrate that the transcriptional functionality of hepatic LXR is preserved during pregnancy and also a decrease in the activity of LXR is likely an essential gestational adaptation which moulds the changes in lipid metabolism during this complex physiological period.

However, another important observation from this study is that although pharmacological activation of LXR during pregnancy was able to raise the absolute concentrations of total and HDL-cholesterol in serum, these lipids exhibited a similar pattern of fluctuation as the one detected during normal pregnancy and dropped on gestational day 10. Similarly, the mRNA levels of hepatic LXR target genes preserved their gestational pattern of variation in the presence of T0901317: a rise on day 7 followed by a decrease from day 10 onwards. These data suggest that there could be other molecular pathways which collaborate with LXR to control lipid homeostasis during mouse pregnancy.

Although the data presented in this document strongly indicate that the transcriptional activity of hepatic LXR changes during pregnancy and that this influences the gestational adaptations in cholesterol metabolism, the exact molecular mechanism modulating the transcriptional capacity

of this nuclear receptor throughout gestation remain elusive. Moreover, our present data does not exclude the possibility that pregnancy could signal the alterations in lipid homeostasis and the changes in the expression of LXR target genes independently of LXR itself. To address these uncertainties, we are in the process of investigating how pregnancy affects lipid metabolism in the absence of Lxrα/β. The results from these studies should allow us to conclusively determine whether the pregnancy environment signals via LXR to induce changes in key metabolic pathways that promote the gestational adaptations in lipid metabolism. Knowledge of the precise signalling pathways that are responsible for the regulation of cholesterol metabolism during pregnancy is of crucial necessity in light of the fact that abnormalities in cholesterol abundance throughout gestation have a profound effect on the longterm health of the developing fetus (Linck et al., 2000, Edison et al., 2007, Napoli et al., 1997, Papacleovoulou et al., 2013).

3.4.2 Study limitations

The main limitation of our study stemmed from the inherent differences in mouse and human lipid metabolism, namely the distribution of cholesterol among lipoprotein classes: whereas humans transport cholesterol predominantly in the form of LDL, mice carry most of their cholesterol in HDL particles (Vergnes et al., 2004). Our results underscored these disparities by showing that while humans develop marked hypercholesterolemia as gestation progresses (Alvarez et al., 1996), the concentrations of total-, HDL- and LDL-cholesterol in the serum are reduced during murine pregnancy (Figure 3.2). To circumvent this limitation, mice could be fed with a cholesterol-enriched diet following the identification of a copulatory plug in order to try and recapitulate the gestational rise in circulating cholesterol levels.

Since oxysterols are physiological regulators of LXR signalling it is essential to know whether pregnancy affects human and murine oxysterol homeostasis both quantitatively and qualitatively (e.g. enrichment of oxysterol species which have lower affinity for LXR or increased availability of oxysterol derivative such sulphated oxysterols which inhibit the activity of this nuclear receptor). However, extraction and quantification of plasma oxysterols employs an intricate methodology and specialised laboratory equipment. Specifically, oxysterol analysis has been recognised as a unique challenge due to the minimal solubility of these molecules, their sequestration into lipoproteins, dramatic differences in the levels of different sterol species (e.g. esterified versus non-esterified sterols) and the interference of other lipids (such as triglycerides and phospholipids) (McDonald et al., 2012). Due to the time constraints of the project and complex methodology of the experiment, oxysterol quantification during human and murine pregnancy has not been performed despite the fact the obtained results would be invaluable in understanding how LXR signalling changes in the context of pregnancy.

Moreover, in order examine how LXR signalling adapts to gestational hypercholesterolemia in order to limit the excessive uptake of sterols into extrahepatic tissues, we aimed to isolate peripheral blood monocytes from women in their third trimester of pregnancy as well as non-pregnant control women. However, due to time constraints, this study has not yet been completed.

Furthermore, another limitation of our study arises from the fact that at present there are no commercially available isoform-specific LXR agonists. Given that we were attempting to activate hepatic LXR, and LXRα is the dominant isoform of this nuclear receptor in the liver, ideally we should have used a ligand specific to this isoform; T0901317 was employed instead, a compound which activates both LXRs. It has to be noted that although T0901317 is a potent LXR agonist, there are conflicting reports suggesting that this molecule is also able to bind and activate the retinoid X receptor (Mitro et al., 2007) as well as FXR (Houck et al., 2004) (these data contradict the results initially published by (Schultz et al., 2000)). However, it has been shown that the affinity of T0901317 for these two nuclear receptors is very low therefore it is expected that T0901317 alters lipid homeostasis during mouse pregnancy in an LXR-dependent manner.

Also, to examine whether the changes in the expression of cholesterol homeostatic genes in the livers of pregnant mice results from alterations in the occupancy of their promoter region, chromatin immunoprecipitation studies need to be performed. However, the success of these experiments depends largely on the availability of validated highly-specific antibodies. In future, chromatin immunoprecipitation studies will be performed to test whether the downregulated expression of LXR targets in the livers of pregnant mice results from the reduced recruitment of the LXR co-activators PGC1α and PGC1β to the promoter regions of these genes. Moreover, it will be interesting to test whether pregnancy alters lipid metabolism by modifying the ability of LXRα/β to bind to the promoters of its targets.

Moreover, to conclusively determine whether the pregnancy environment signals via LXR to induce changes in key metabolic pathways that promote the gestational adaptations in lipid metabolism, pregnancy studies using Lxrα/β-deficient mice will be conducted. The completion of this experiment has been delayed due to the reduced fertility in these the mutants (female Lxrα/β knockout mice present with resistance to follicular fluid meiosis-activating sterol and subsequently reduced oocyte meiosis, symptoms of ovarian hyperstimulation syndrome and reduced uterine contractility due to the accumulation of cholesterol esters in the uterine myometrium; male Lxrα/β knockout mice become progressively more infertile as they age due to sterol-induced abnormalities in the epididymal epithelium, accumulation of cholesterol esters

in Sertoli cells and degeneration of the seminiferous tubules of the testes, hypoandrogenism and impaired spermatogenesis (Beltowski and Semczuk, 2010)). This experiment is of vital importance since it should confirm whether intact LXR signalling is imperative for the maintenance of cholesterol in normal levels during non-complicated pregnancy.

Finally, having hypothesised that, in addition to LXR signalling, there are other key metabolic pathways that change during pregnancy and thereby contribute to the alterations in lipid homeostasis during this period, we examined how the metabolic activities of subcutaneous and visceral white adipose tissues are regulated in non-complicated pregnancy and gestational cholestasis.

Chapter 4

Distinct Contributions of Subcutaneous and Visceral Fat to Lipid Metabolism in Normal Pregnancy and Gestational Cholestasis

4.1 Introduction

Pregnancy is a complex state characterised by changes in maternal nutrient metabolism (Herrera, 2002). These adaptations are necessary in order to ensure a continuous supply of essential metabolites to support the growth and the development of the fetus as well as to provide the mother with sufficient energy stores to meet the demands of pregnancy and prepare for lactation (Herrera, 2002). Lipid metabolism changes progressively throughout gestation and in the third trimester the mother develops hyperlipidaemia, defined by increases in the concentrations of total and lipoprotein-bound cholesterol and triglycerides as a consequence to the increased lipolytic activity in adipose tissue during this period (Alvarez et al., 1996).

Intrahepatic cholestasis of pregnancy (ICP) is a pregnancy-specific liver disorder which is typically diagnosed in the third trimester and is characterised by raised serum bile acids and impaired liver function (Laatikainen and Ikonen, 1977). Women with ICP have an increased risk of developing gestational diabetes and also present with dyslipidaemia comprising significantly raised fasting levels of plasma total triglycerides and increased total and LDL-cholesterol concentrations; circulating levels of HDL-cholesterol are significantly reduced in these women (Dann et al., 2006, Martineau et al., 2015).

White adipose tissue (WAT) functions as a long-term energy repository which actively integrates pathways for nutrient storage and mobilisation in accordance with whole-body energy balance (Rosen and Spiegelman, 2014). Furthermore, WAT is a dynamic endocrine organ that secretes a range of adipokines and cytokines which, in turn, signal to other organs, such as liver, muscle and brain, to regulate their metabolic activity (Rosen and Spiegelman, 2014). Unlike other tissues, white fat develops in multiple discrete locations throughout the body, forming depots that have unique cellular composition, adipogenic potential, lipid metabolism and endocrine profiles (Rosen and Spiegelman, 2014). The most common classification schemes distinguish between subcutaneous (under the skin) and visceral (inside the body cavity) fat; this is an oversimplification arising from the fact that visceral fat accumulation is associated with elevated risk of insulin resistance, type 2 diabetes, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis and overall mortality (Tran and Kahn, 2010). In contrast, fat accumulation predominantly in the subcutaneous compartment is linked to improved insulin sensitivity and a lower risk of developing metabolic syndrome (Snijder et al., 2003).

The adaptations in WAT metabolism during human pregnancy follow a biphasic pattern: the first two trimesters of gestation are centred on triglyceride deposition in maternal adipose tissues whereas in late pregnancy, that rate of fat accrual progressively declines and stored lipids

are hydrolysed in order to fuel the metabolism of the mother and the developing fetus (Alvarez et al., 1996).

Although it is well documented that visceral and subcutaneous fat depots play contrasting roles in the control of lipid and carbohydrate metabolism under conditions of metabolic dysfunction, there is a substantial gap in our knowledge of whether these two depots change differently in response to physiological and cholestatic pregnancy. We hypothesised that visceral and subcutaneous fat depots have unique contributions to the development of gestation-specific adaptations in lipid metabolism and that WAT dysfunction could play a role in the development of dyslipidaemia in pregnancies complicated with ICP. The data presented in this chapter will aim to expand the knowledge on these two topics by describing and comparing the structural, metabolic and endocrine adaptations taking place in visceral and subcutaneous WAT during non-complicated mouse pregnancy and in mouse pregnancy complicated with gestational cholestasis.

4.2 Experimental methodology

To study the contributions of subcutaneous and visceral fat to the changes in lipid metabolism during non-complicated pregnancy and pregnancy complicated with gestational cholestasis, female mice were fed with standard chow diet or chow diet supplemented with 0.5% cholic acid (CA diet; previously described by (Watanabe et al., 2006, Papacleovoulou et al., 2013)) following the identification of a copulation plug (Figure 4.1). Pregnant females were sacrificed on day 14 of gestation. Tissues and sera were collected also from CRM-fed virgin controls and non-pregnant mice fed with a CA diet for a period of 14 days. Whole tissue samples were used for the lipid profiling, protein expression analysis and histological studies. Primary mature adipocytes were isolated for gene expression studies.

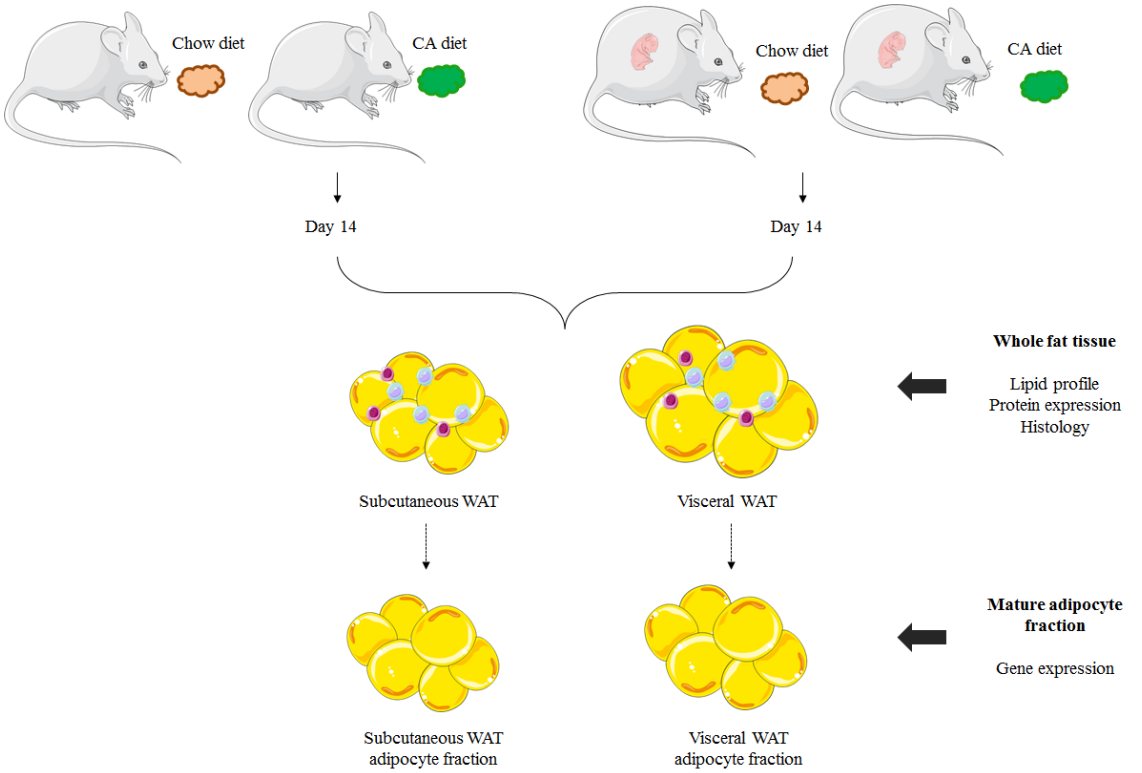


FIGURE 4.1: Schematic summary of the methodological approach to study the contributions of subcutaneous and visceral fat to the changes in lipid metabolism during non-complicated pregnancy and pregnancy complicated with gestational cholestasis.

4.3 Results

4.3.1 Subcutaneous and visceral WAT expansion during non-complicated pregnancy

Subcutaneous and visceral fat depots differ in their mechanisms for tissue expansion in response to lipid overload: subcutaneous WAT grows predominantly by hyperplasia, whereas visceral fat enlarges through hypertrophy. Therefore, we studied adipose tissue expansion in these two depots during advanced murine pregnancy (day 14pc). Virgin female mice were used as non-pregnant controls.

Initial characterisation revealed that the fat pad weight and triglyceride content of both subcutaneous and visceral WAT increased significantly in pregnant as compared to non-pregnant mice (Figure 4.2). Morphological analysis of haematoxylin and eosin-stained adipose tissue sections showed that the mean adipocyte area of both depots was higher in pregnant animals than in non-pregnant ones. However, a more detailed study of adipocyte distribution suggested that pregnancy increases the abundance of subcutaneous WAT adipocytes whose area is between 1000-4000μm^2 and the enrichment of visceral WAT adipocytes whose area is between 2000-10000μm^2; at the same time the abundance of adipocytes whose area is less than 1000μm^2 and 2000 μm^2 in subcutaneous and visceral fat respectively was lower in pregnant mice than in non-pregnant controls.

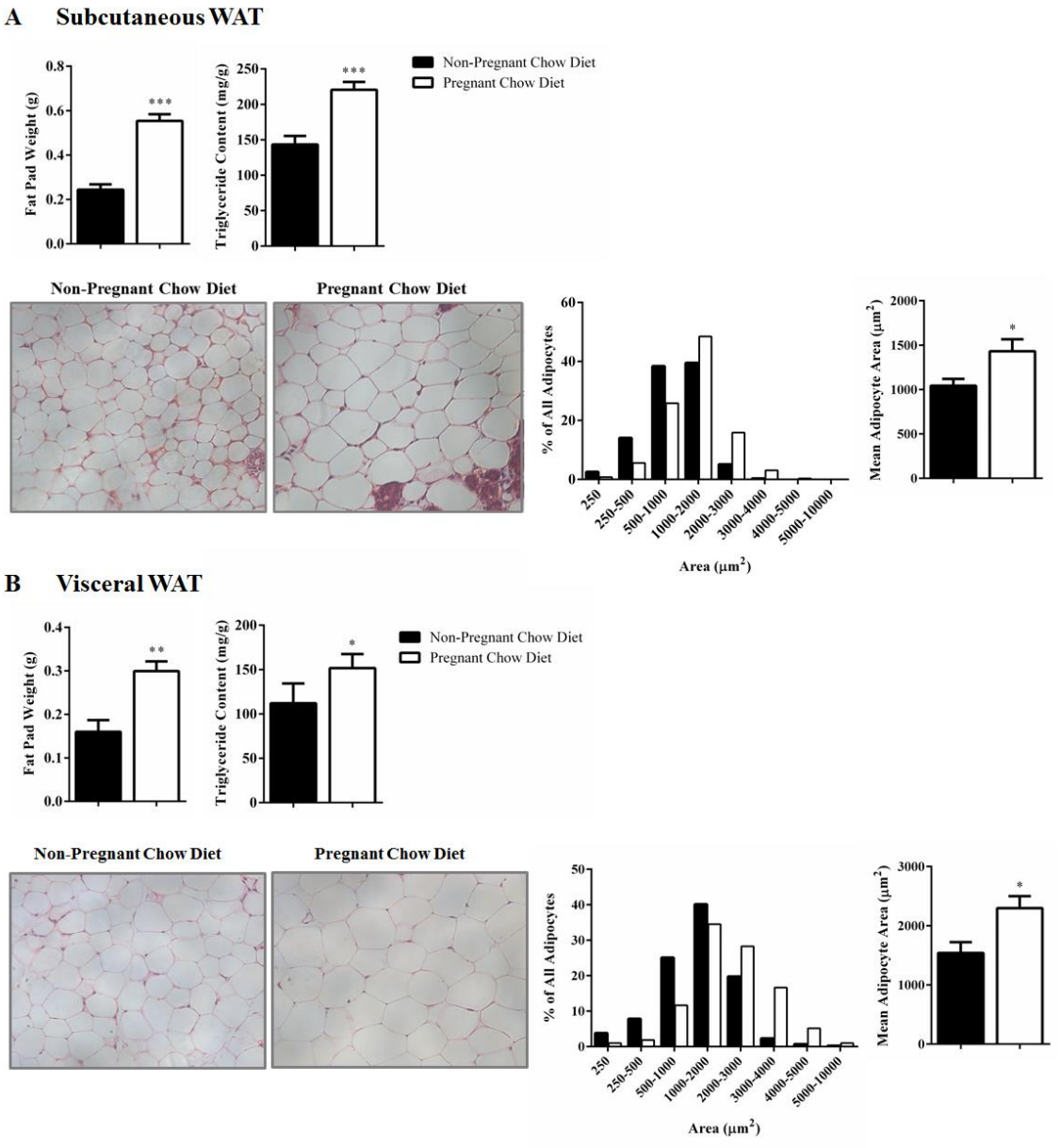


FIGURE 4.2: Impact of pregnancy on the fat pad weight, triglyceride content and adipocyte size of adipose tissue. Wild-type mice fed with standard chow diet were sacrificed on day 14 of pregnancy; non-pregnant mice were used as controls. **A**. Subcutaneous WAT. **B**. Visceral WAT. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of pregnant versus non-pregnant group. Data were analysed using unpaired two-tailed t-test.

4.3.2 Impact of pregnancy on adipose tissue lipogenesis

Adipose tissue is an organ highly adapted to storing surplus dietary fatty acids in the form of triglycerides and subsequently releasing them under conditions of negative energy balance in the body. In advanced pregnancy, WAT metabolism undergoes adaptations that are vital to ensure the increased mobilisation of lipid deposits; during this period, the rate of triglyceride accrual is diminished. Subsequently, we studied the impact of pregnancy on PPARγ-controlled lipogenic pathways in subcutaneous and visceral WAT.

Although WAT is predominantly constituted of mature adipocytes, it also comprises stromal vascular cells such as fibroblasts, macrophages and mammary cells (especially predominant in the subcutaneous fat of pregnant mice). PPAR transcriptome is expressed in all of the different adipose tissue cell types and therefore in order to examine the impact of pregnancy specifically on mature adipocytes, these cells were isolated and subjected to mRNA profiling.

The results from our study indicated that the transcript abundance of Pparγ2 itself as well as its lipogenic targets Ap2, Lpl, Pepck and Glut4 was significantly reduced in the adipocyte fraction of both subcutaneous and visceral fat of pregnant mice as compared to non-pregnant controls (Figure 4.3).

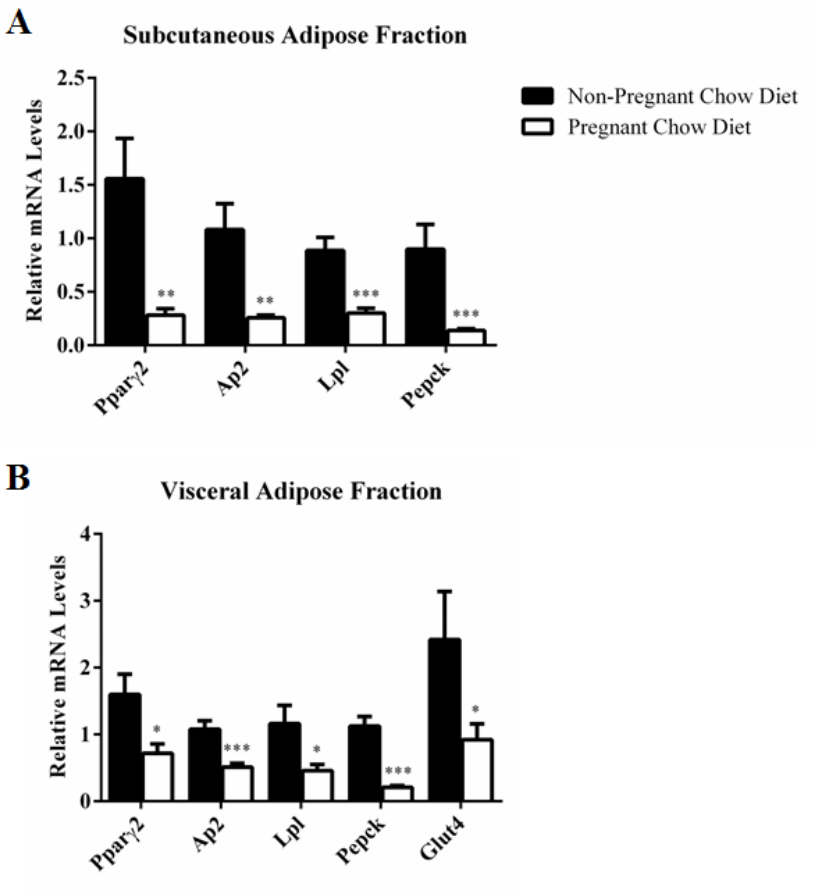


FIGURE 4.3: Impact of pregnancy on lipogenesis in subcutaneous and visceral WAT. Wild-type mice fed with standard chow diet were sacrificed on day 14 of pregnancy; non-pregnant mice were used as controls. Relative mRNA levels were quantified using RT-PCR. **A**. Gene expression of Pparγ2, Ap2, Lpl and Pepck in the adipose fraction of subcutaneous fat. **B**. Gene expression of Pparγ2, Ap2, Lpl, Pepck and Glut4 in the adipose fraction of visceral fat. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of pregnant versus non-pregnant group. Data were analysed using unpaired two-tailed t-test.

4.3.3 Subcutaneous and visceral WAT endocrine profiles during non-complicated pregnancy

Adipose tissue regulates glucose and lipid homeostasis not only by controlling the availability of circulating free fatty acids but also by secreting signalling molecules, cytokines and adipokines, which modulate the metabolic activity of key tissues such as liver and skeletal muscle. To determine if pregnancy affects differently the endocrine profiles of subcutaneous and visceral WAT we profiled the abundance of inflammatory markers in these fat depots in pregnant mice and non-pregnant controls.

The results from this study indicated that the protein abundance of retinol-binding protein 4 (RBP4), C-recative protein (CRP), interleukin 11 (IL11), Endocan 1, Fetuin A and Dipeptidyl peptidase 4 (DPP4) were raised in the subcutaneous WAT of pregnant mice (Table 4.1); the levels of these factors were minimally altered in visceral fat during murine pregnancy.

TABLE 4.1: Impact of pregnancy on the endocrine profiles of adipose tissue depots

	Subcutaneous WAT	Visceral WAT
Pro-inflammatory factors		
Retinol-binding protein 4	2.69	1.30
C-reactive protein	2.21	1.04
IL-11	2.09	not detected
Endocan 1	1.94	1.79
Fetuin A	1.78	0.97
Dipeptidyl peptidase 4	1.65	1.02

Fold changes in the protein levels of adipokines in subcutaneous and visceral WAT in pregnant mice as compared to non-pregnant controls (normalised to 1). Targets were immunodetected in pooled adipose tissue extracts (6 mice per group).

4.3.4 Impact of cholestasis on murine lipid profiles

ICP is a human pregnancy disease associated with raised serum levels of bile acids and deranged lipid homeostasis (Martineau et al., 2015). To determine whether bile acids overload in the serum could also affect lipid metabolism during murine pregnancy, female mice were fed with a standard chow diet supplemented with 0.5% of cholic acid (CA diet) for a period of 14 days following the identification of a copulatory plug. Pregnancy-matched mice fed with normal chow as well virgin mice fed with chow/ CA diet for a period of 14 days were used as controls.

According to the results from our study, pregnant mice fed with a CA diet had serum triglyceride and free fatty acid concentrations that were significantly higher than the ones detected in pregnant controls (Figure 4.4A). In contrast, CA administration to non-pregnant mice significantly reduced the levels of triglycerides in the sera of these animals without affecting the abundance of free fatty acids (Figure 4.4B).

Moreover, bile acid feeding reduced the levels of HDL-cholesterol and increased the concentrations of LDL-cholesterol in the sera of pregnant and non-pregnant ($p \leq 0.05$); circulating total cholesterol levels were not significantly altered by CA administration.

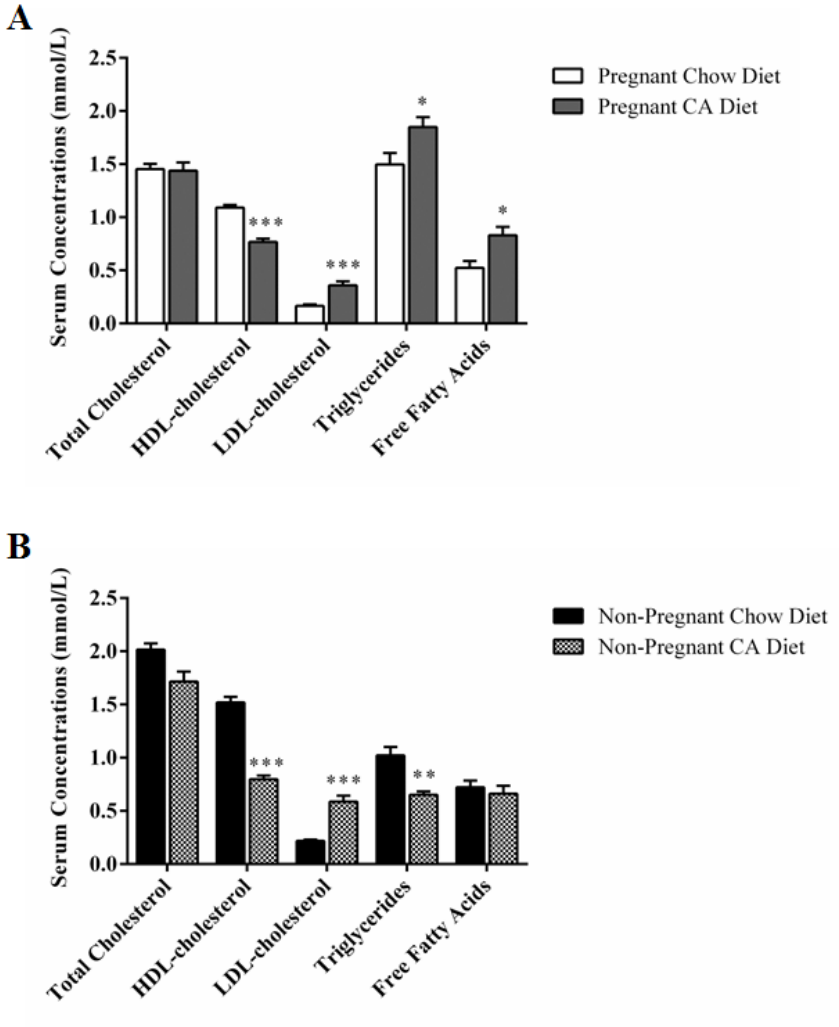


FIGURE 4.4: Impact of raised bile acids on the serum lipid profiles of pregnant and non-pregnant mice. Wild-type mice fed with standard chow/ CA diet following the identification of a copulatory plug were sacrificed on day 14 of pregnancy; diet-matched non-pregnant mice were used as controls. Serum concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides and free fatty acids were measured. **A**. Serum lipid profiles of pregnant mice. **B**. Serum lipid profiles of non-pregnant mice. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of CA-fed versus chow-fed group. Data were analysed using unpaired two-tailed t-test.

4.3.5 Impact of bile acid feeding during pregnancy on white adipose tissue composition

CA feeding increases the circulating levels of triglycerides and free fatty acids only in pregnant mice and adipose tissue regulates the availability of these lipids in the serum, suggesting that bile acid overload during pregnancy could impair WAT function during pregnancy thereby precipitating the development of metabolic dysfunction. Since subcutaneous and visceral fat play contrasting roles under conditions of metabolic derangements, we studied these two depots in parallel.

Our data indicated that the fat pad weight and triglyceride content of both subcutaneous and visceral WAT were significantly reduced in pregnant mice fed with a CA diet as compared to chow-fed controls (Figure 4.5). Morphological analysis of haematoxylin and eosin-stained adipose tissues sections showed that the mean adipocyte area of both depots was lower in pregnant CA-fed mice than in their chow-fed counterparts. Moreover, a more detailed study of adipocyte distribution suggested that bile acid administration reduces the abundance of subcutaneous WAT adipocytes whose area is between 1000-3000μm^2 and the enrichment of visceral WAT adipocytes whose area is between 3000-10000μm^2; at the same time the abundance of adipocytes whose area is less than 1000μm^2 and 2000 μm^2 in subcutaneous and visceral fat respectively was higher in CA-fed pregnant mice than in the chow-fed controls.

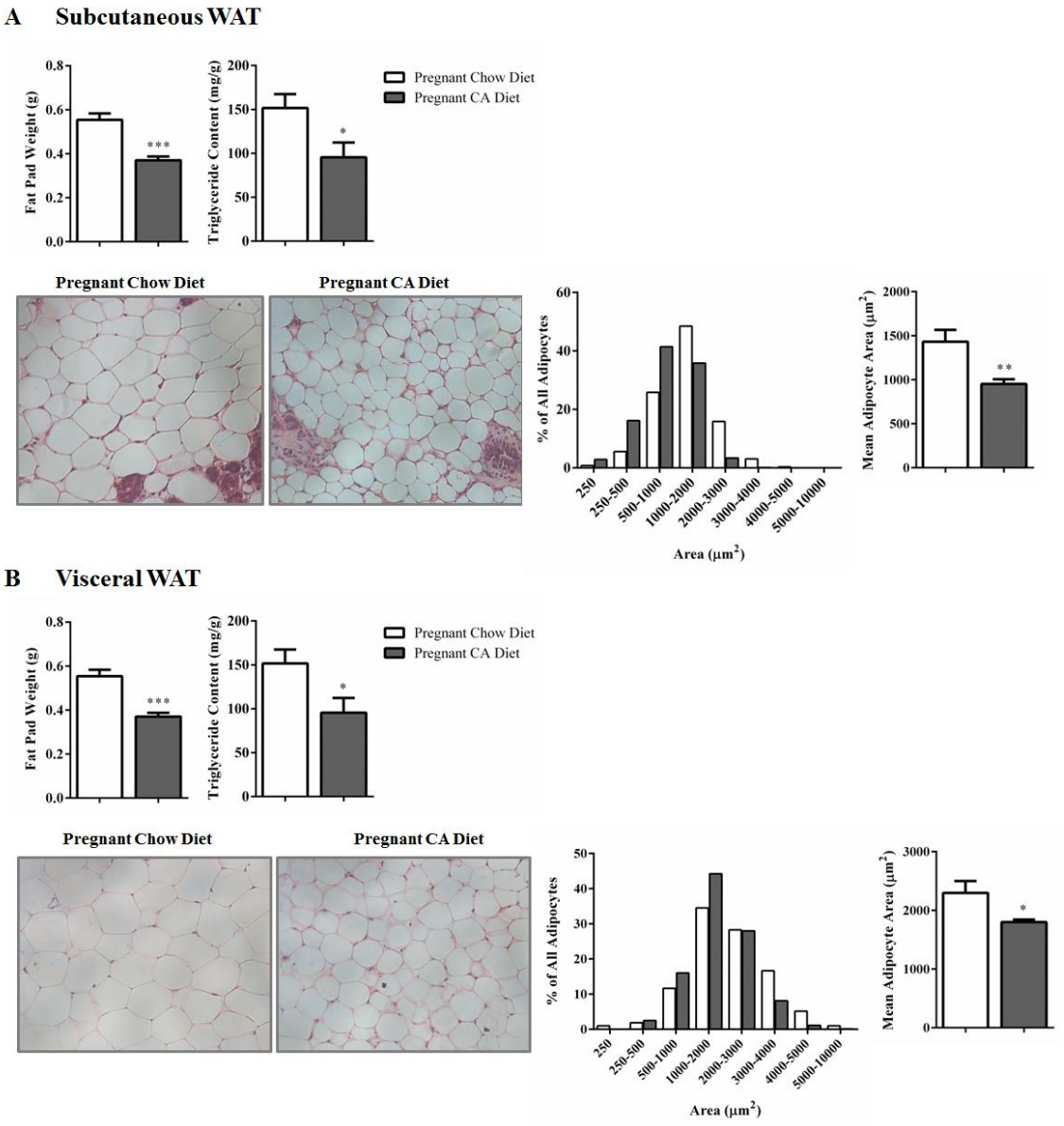


FIGURE 4.5: Impact of bile acid feeding during pregnancy on the fat pad weight, triglyceride content and adipocyte size of WAT. Wild-type mice fed with standard chow/ CA diet following the identification of a copulatory plug were sacrificed on day 14 of pregnancy; diet-matched non-pregnant mice were used as controls. **A**. Subcutaneous WAT. **B**. Visceral WAT. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of CA-fed versus chow-fed group. Data were analysed using unpaired two-tailed t-test.

4.3.6 Impact of bile acid feeding during pregnancy on adipose tissue lipogenesis

The fact that the triglyceride content of both subcutaneous and visceral fat was reduced as a consequence to CA administration, despite the abundance of circulating triglycerides and fatty acids, indicted that supraphysiological levels of bile acids could impair the ability of adipose tissue to internalise and store serum lipids. Therefore, we examined the impact of bile acid feeding during murine pregnancy on PPARγ-controlled lipogenic pathways in subcutaneous and visceral WAT.

The results from our study indicated that the transcript abundance of Pparγ2 itself as well as its lipogenic targets Ap2, Lpl, Pepck and Glut4 was significantly reduced in the adipocyte fraction of both subcutaneous and visceral fat of CA-fed pregnant mice as compared to chow-fed controls (Figure 4.6).

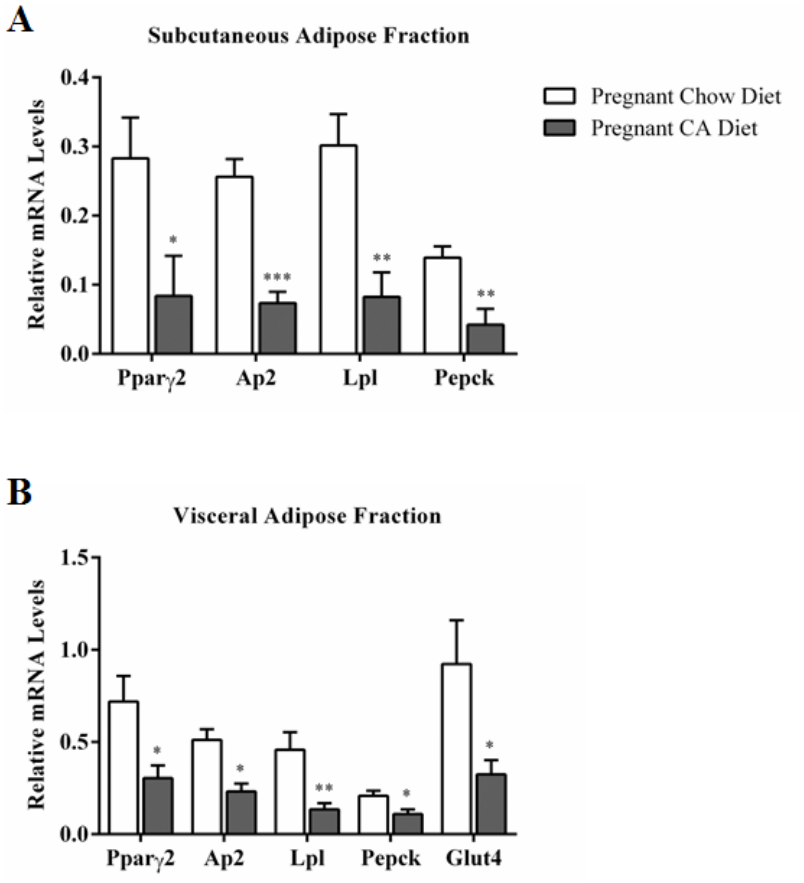


FIGURE 4.6: Impact of bile acid feeding during pregnancy on lipogenesis in subcutaneous and visceral WAT. Wild-type mice fed with standard chow/ CA diet following the identification of a copulatory plug were sacrificed on day 14 of pregnancy; diet-matched non-pregnant mice were used as controls. Relative mRNA levels were quantified using RT-PCR. **A**. Gene expression of Pparγ2, Ap2, Lpl and Pepck in the adipose fraction of subcutaneous fat. **B**. Gene expression of Pparγ2, Ap2, Lpl, Pepck and Glut4 in the adipose fraction of visceral fat. Results are represented as mean ± SEM (n=6-8) *p≤0.05, **p ≤ 0.01, ***p ≤ 0.001, comparison of CA-fed versus chow-fed group. Data were analysed using unpaired two-tailed t-test.

4.3.7 Impact of bile acid feeding during pregnancy on the endocrine profiles of adipose tissue depots

WAT is a dynamic endocrine organ which secretes hormones and signals to modulate its own metabolism as well as the metabolism of peripheral tissues such as skeletal muscle and brain. Since the energy storage potential of WAT is impaired as a consequence of the bile acid overload in gestational cholestasis, we theorised that the endocrine signalling of this organ could also be altered under these conditions thereby contributing to the local and whole-body metabolic dysfunction. An adipokine array was employed to determine the impact of bile acid overload during pregnancy on the protein levels of endocrine factors in subcutaneous and visceral fat.

The obtained data indicated that gestational cholestasis reduces the abundance of pro-inflammatory factors in white fat. Moreover, this effect was depot-specific since CA-fed pregnant mice presented with reduced levels of RBP4 and plasminogen activator inhibitor 1 (PAI-1) in subcutaneous WAT whereas the factors decreased in visceral fat were RBP4, DPP4, Fetuin A and Endocan 1 (Table 4.2).

TABLE 4.2: Impact of CA feeding during pregnancy on the endocrine profiles of adipose tissue depots

Subcutaneous WAT		Visceral WAT	
Retinol-binding protein 4	0.85	Retinol-binding protein 4	0.80
Plasminogen activator inhibitor 1	0.73	Dipeptidyl peptidase 4	0.83
		Fetuin A	0.82
		Endocan 1	0.60

Fold changes in the protein levels of adipokines in subcutaneous and visceral WAT in pregnant CA-fed mice as compared to chow-fed controls (normalised to 1). Targets were immunodetected in pooled adipose tissue extracts (6 mice per group).

4.3.8 Impact of bile acid administration to non-pregnant mice on adipose tissue function

Unlike pregnant mice, non-pregnant mice fed with a CA-supplemented diet for a period of 14 days did not present with raised levels of serum free fatty acids and the circulating levels of triglycerides in their sera were in fact lower than the ones detected in chow-fed controls (Figure 4.4B). To confirm that bile acids do not compromise the lipid storage function of white fat in non-pregnant mice, subcutaneous and visceral WAT depots were examined.

The results from our study showed that feeding non-pregnant mice with a CA-supplemented diet did not have an effect on the fat pad weight and triglyceride content of subcutaneous and visceral WAT (Figure 4.7A). Moreover, the mRNA levels of Pparγ2 itself as well as its lipogenic targets Ap2, Lpl, Pepck and Glut4 were not significantly altered in the adipocyte fraction of both fat depots in CA-fed non-pregnant mice as compared to chow-fed controls (Figure 4.7B).

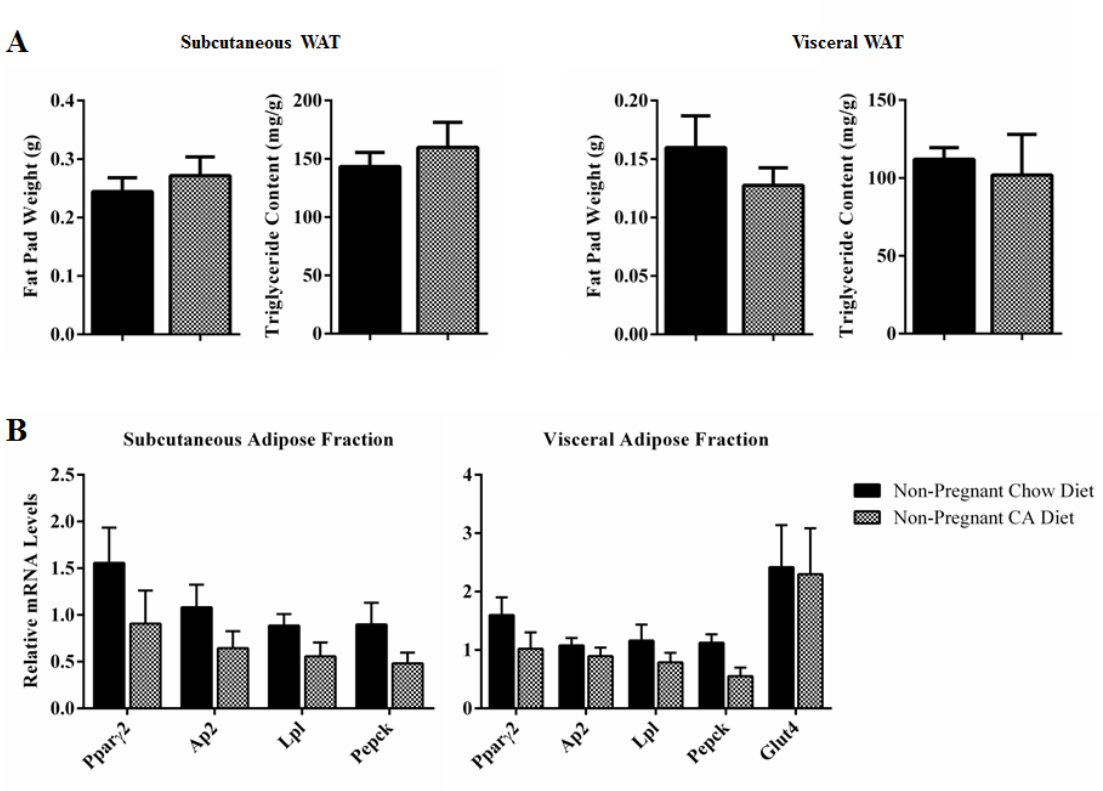


FIGURE 4.7: Impact of bile acid administration to non-pregnant mice on adipose tissue function. Wild-type mice fed with standard chow/ CA diet following the identification of a copulatory plug were sacrificed on day 14 of pregnancy; diet-matched non-pregnant mice were used as controls. **A**. Fat pad weight and triglyceride content of subcutaneous and visceral WAT. **B**. Gene expression of Pparγ2, Ap2, Lpl, Pepck and Glut4 in the adipose fraction of subcutaneous and visceral fat. Relative mRNA levels were quantified using RT-PCR. Results are represented as mean ± SEM (n=6-8).

In conclusion, the data described in this chapter demonstrated that in advanced murine pregnancy adipose tissue lipogenesis is reduced in both subcutaneous and visceral WAT. Although both of these depots expanded in the course of gestation in order accommodate triglyceride accrual, there was increased inflammation specifically in subcutaneous WAT; in contrast, visceral fat remained quiescent.

Moreover, maintenance of supraphysiological levels of bile acids for a period of 14 days had unique effects on lipid metabolism in non-pregnant and pregnant mice. Although it decreased HDL-cholesterol and elevated LDL-cholesterol levels in both of these animal groups, CA feeding reduced serum triglyceride concentrations in non-pregnant rodents. Bile acid administration has no effect on circulating free fatty acid levels, adipose tissue expansion and lipogenesis in these animals.

On the other hand, feeding pregnant mice with a CA-supplemented diet raised their serum triglyceride and free fatty acid levels and reduced adipose tissue lipogenesis. Gestational cholestasis also decreased white fat inflammation in a depot-specific manner and interfered with WAT remodelling and expansion. Failure of fat to grow and store surplus lipids that normally accumulate during pregnancy is likely to precipitate the development of dyslipidaemia.

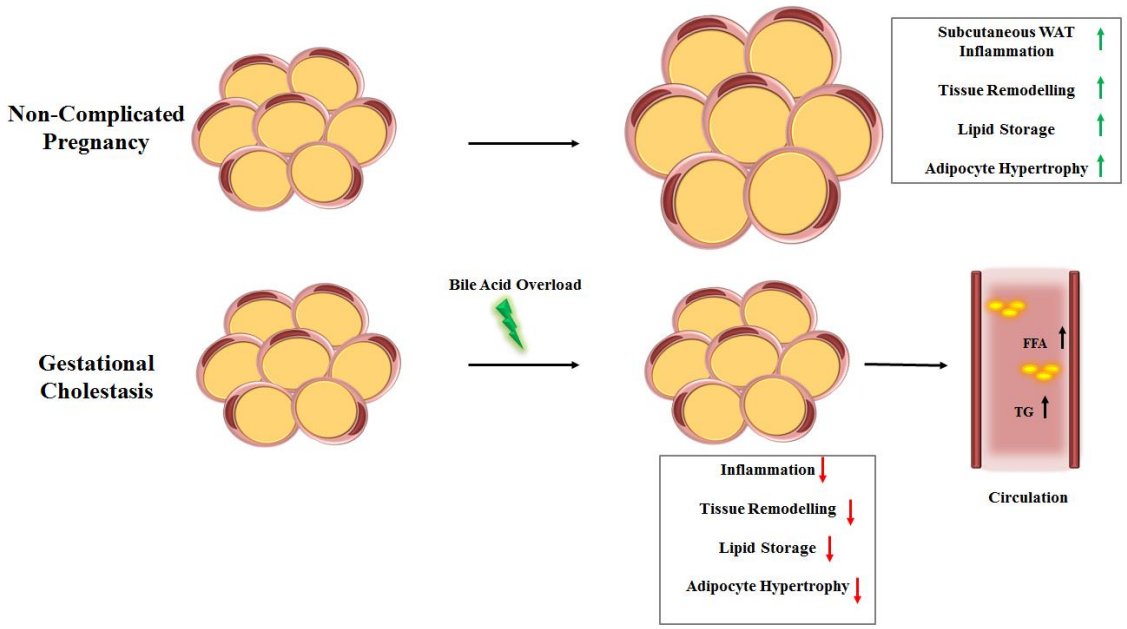


FIGURE 4.8: Summary of the impact of non-complicated pregnancy and gestational cholestasis on white adipose tissue function. WAT, white adipose tissue; FFA, free fatty acids; TG, triglycerides.

4.4 Discussion

4.4.1 Subcutaneous and visceral WAT are differentially regulated during advanced mouse pregnancy

Pregnancy is an intricate biological condition associated with profound adaptations in the nutrient metabolism of the mother; these adjustments are of vital importance to ensure that the fetus is supplied with sufficient energy and biochemical substrates to support its growth and development, and also to fulfil the raised physiological demands of the mother (Herrera, 2002). Lipid metabolism adapts continuously throughout pregnancy and during advanced gestation the mother develops hyperlipidaemia, defined by increases in the concentrations of total and lipoprotein-bound cholesterol and triglycerides, as a consequence of the insulin resistance and increased lipolytic activity in adipose tissue during this period (Alvarez et al., 1996). Adipose tissue regulates lipid homeostasis directly, by controlling the availability of free fatty acids and glucose, and indirectly, by dynamically secreting endocrine signals which in turn modulate the metabolic activity of other organs (Rosen and Spiegelman, 2014). Given that subcutaneous and visceral fat play contrasting roles in the management of nutrient homeostasis, we examined if these two depots have differential contributions to the gestational adaptations in lipid metabolism.

The results from our study demonstrate for the first time that although both depots expand in order to accommodate the increased triglyceride accrual during murine pregnancy, subcutaneous WAT exhibits an inflammatory phenotype on day 14 of non-complicated mouse pregnancy while visceral fat remains quiescent. Specifically, the protein expression of RBP4, CRP, IL11, Endocan 1, Fetuin A and DPP4 were raised in the subcutaneous WAT of pregnant mice, whereas their abundance in the visceral compartment was generally unchanged.

RBP4 is key adipose tissue-derived factor that induces insulin resistance indirectly not only by stimulating the production of pro-inflammatory cytokines in fat macrophages via pathways involving Toll-like receptor and c-Jun N-terminal kinase (JNK) signalling (Norseen et al., 2012) but also by stimulating JAK/STAT5 cascades (Berry et al., 2011). A recent study has demonstrated that RBP4 directly activates antigen-presenting cells in JNK-dependent manner and thereby increases the infiltration of WAT with immune cells (Moraes-Vieira et al., 2014). Moreover, overexpression of this retinol transporter promotes the development of a pro-inflammatory phenotype in alternatively-activated macrophages (Moraes-Vieira et al., 2014). The potency of this adipokine is further illustrated by *in vivo* experiments where transplantation of RBP4-activated immune cells into lean mice is sufficient to elicit adipose tissue inflammation and insulin resistance (Moraes-Vieira et al., 2014). Based on this data, we have concluded that

subcutaneous WAT is crucial for the development of pregnancy-associated insulin resistance since it overproduces RBP4 during advanced gestation in mice.

Similarly, CRP is an acute-phase protein whose plasma concentrations are very low under healthy conditions (Pepys and Hirschfield, 2003, Anty et al., 2006). The plasma half-life of this peptide is approximately 18 hours; therefore CRP is employed as a non-specific marker of inflammation since any elevations in its circulating levels are a direct reflection of raised synthesis in response to pathological processes (Pepys and Hirschfield, 2003, Anty et al., 2006). CRP is produced by adipose tissue and its levels are also strongly associated with obesity and metabolic syndrome-related diseases, including insulin resistance, diabetes mellitus, and hyperlipidaemia (Pepys and Hirschfield, 2003, Anty et al., 2006). Interestingly, studies in healthy post-menopausal women receiving a hormone replacement therapy have shown that administration of estrogen increases the basal concentrations of CRP in the absence of any tissue-damaging inflammation (Ridker et al., 1999). Consequently, it is plausible that reproductive hormones, in particular estrogen, could play a role in the induction of low-grade inflammation in subcutaneous fat as an essential gestational adaptation that contributes to the adjustments in lipid metabolism during non-complicated pregnancy.

Moreover, IL11 is a member of the interleukin 6 family of cytokines and it induces inflammation by stimulating Janus kinase signalling and raising the expression of STAT1 and STAT3 transcription factors (Tenney et al., 2005, Ernst et al., 2008). This cytokine also contributes to the pro-inflammatory phenotype of subcutaneous fat in pregnant mice.

Additionally, Endocan 1 is a proteoglycan naturally secreted by vascular endothelial cells which is highly regulated by the abundance of inflammatory cytokines (Delehedde et al., 2013). *In vivo* and *in vitro* studies have demonstrated that Endocan 1 is a potent molecule which is able to elicit severe inflammatory response and therefore it could be essential for the vascular contribution to organ-specific inflammation (Delehedde et al., 2013). In agreement with this, it has been reported that the circulating levels of Endocan are significantly elevated in patients suffering from sepsis while neutralisation of Endocan with the help of an antibody reduces sepsis-induced mortality (Lee et al., 2014). Based on these data, it is conceivable that the rise in the protein levels of this proteoglycan in subcutaneous WAT could either be a direct result from the increase in local inflammation in this tissue or it could contribute to the overall pro-inflammatory milieu in the depot.

Furthermore, Fetuin A is a glycoprotein predominantly secreted by the liver, but also produced by white adipose tissue (Hennige et al., 2008). *In vivo* studies have demonstrated that Fetuin A potently induces inflammation by increasing the expression of pro-inflammatory cytokines in

mouse fat; it is also able to reduce the expression of Pparγ in murine WAT (Hennige et al., 2008). Moreover, this glycoprotein promotes insulin resistance by repressing the activity of the insulin receptor tyrosine kinase in liver and skeletal muscle (Srinivas et al., 1993). In agreement with this observation, mice devoid of Fetuin A present with improved insulin sensitivity and resistance to diet-induced obesity (Mathews et al., 2002). Studies of the gene encoding Fetuin A in humans have suggested that this glycoprotein is a key determinant of insulin action in adipocytes (Dahlman et al., 2004). Consequently, the increase in the protein levels of Fetuin A in the subcutaneous fat of pregnant mice could promote inflammation and contribute to the development of insulin resistance characteristic of advanced gestation.

Finally, DPP4 is a ubiquitously expressed serine protease which localises at the apical membrane of cells where it cleaves the N-terminal dipeptides from a variety of substrates including chemokines, growth factors and inflammatory cytokines (Lambeir et al., 2003). It also exists in a soluble plasma form, which is catalytically active despite the fact that it lacks the cytoplasmic tail and the transmembrane region of the transmembrane protein (Lambeir et al., 2003). DPP4 expression in adipose tissue is increased in obesity and its circulating levels correlate directly with hallmark symptoms of metabolic syndrome such as plasma hypertriglyceridemia and fat cell volume (Lamers et al., 2011). Moreover, DPP4 is predominantly secreted by visceral fat and it impairs insulin signalling in a paracrine and hormonal manner (Lamers et al., 2011). Also, exogenous administration of DPP4 is able to induce the expression and secretion of pro-inflammatory cytokines (Wronkowitz et al., 2014) while inhibition of this adipokine decreases monocyte activation and chemotaxis (Shah et al., 2011).

All of these data convincingly demonstrate during advance murine pregnancy subcutaneous WAT mounts an inflammatory phenotype which contributes to the development of insulin resistance during this gestational period. These results are novel and exciting in the light of the fact that classically it is the visceral fat that is considered prone to developing inflammation under conditions of lipid overload.

One possible explanation of this phenotype is that during pregnancy visceral WAT is protected against inflammation. In agreement with this theory, a recent study has demonstrated that during late pregnancy mice with dietary-induced obesity present with reduced visceral fat hypertrophy, ameliorated inflammation and improved glucose tolerance; it has been proposed that ERα signalling mediates these beneficial effects on visceral WAT (Pedroni et al., 2014). However, peripheral adiposity was not improved in these mice (Pedroni et al., 2014). Moreover, since inflammatory signalling plays a key role in the induction in labour and visceral fat is physically

associated with the fetoplacental unit, it is possible that dampened inflammatory response in this depot serves to protect against preterm delivery.

Furthermore, it is well established that WAT inflammation potently induces the hydrolysis of triglycerides stored in fat tissue and this effect is independent of catecholamine-induced lipolysis. For instance, it has been demonstrated that TNFα augments adipocyte lipolysis by stimulating MAPK and JNK signalling cascades that raise cAMP abundance and activate PKA-dependent lipolysis (described in Figure 1.5) (Zhang et al., 2002). Also, TNFα promotes the release of free fatty acids from lipid droplets by supressing the expression of G0S2, an endogenous inhibitor of ATGL (Jin et al., 2014). In addition, adipose tissue inflammation controls lipid homeostasis by downregulating PPARγ signalling and lipogenesis. Specifically, it has been shown that TNFα is able to inhibit the activity of this nuclear receptor by regulating its transcription, translation, posttranslational modifications and protein turnover (Guilherme et al., 2008). Based on these data, it is conceivable that the increase in inflammation in subcutaneous WAT during advanced mouse pregnancy could play an essential role in the development of the two hallmark adaptations in lipid metabolism during this gestational period: limited fat accrual and enhanced triglyceride mobilisation. In agreement with this, we have observed that the expression of Pparγ2 and its targets, which are essential for the assimilation of circulating free fatty acids and their deposition into lipid droplets in mature adipocytes, is reduced in subcutaneous fat during advanced murine pregnancy.

On the other hand, PPARγ-dependent lipogenesis is also reduced in the visceral fat of pregnant mice. Since no changes in the adipokine milieu of this depot were detected, it is likely that alternative mechanisms, independent of inflammation, contribute to this adjustment. Further studies are necessary to elucidate the molecular pathways that downregulate lipogenesis in this tissue.

In conclusion, the results from our study demonstrate for the first time that although both depots expand as a consequence to the pregnancy-induced deposition of energy substrates, subcutaneous and visceral WAT are differentially regulated during advanced gestation in the mouse. Subcutaneous WAT develops an inflammatory phenotype that is likely to contribute to the enhanced hydrolysis of stored lipids during advanced gestation while visceral fat remains quiescent. Further studies are needed in order to determine whether these differences have a functional impact on the potential of these two depots to release free fatty acids in order to fuel the metabolism of the mother and support the growth of the developing fetus.

4.4.2 Gestational cholestasis impairs adipose tissue lipogenesis and thereby precipitates the development of dyslipidaemia

ICP is a pregnancy-specific disease whose hallmark characteristic is the accumulation of supraphysiological concentrations of bile acids in the serum of affected women. Recent studies have also demonstrated that ICP is associated with metabolic dysfunction, namely gestational dyslipidaemia (raised plasma levels of total cholesterol, LDL-cholesterol, triglycerides and free fatty acids, and reduced HDL-cholesterol concentration) and impaired glucose homeostasis (Martineau et al., 2015). However, the exact pathophysiological mechanisms precipitating the derangement of lipid homeostasis in cholestatic women are still unclear.

There are no published reports on the impact of ICP on hepatic lipid metabolism. However, a recent study from our lab has shown that bile acid overload in late pregnant mice increases the cholesterol content of the liver without affecting the abundance of triglycerides and free fatty acids in this organ (Papacleovoulou et al., 2013). Hepatic hypercholesterolemia, as a consequence of enhanced absorption of dietary sterols or elevated *de novo* biosynthesis, could explain the increase in serum total and LDL-cholesterol levels (Figure 3.2). However, the increase in the circulating levels of triglycerides and free fatty acids observed in women suffering from ICP, together with the fact that white adipose tissue stringently controls the availability of these two lipid molecule species, strongly suggest the WAT function could be altered by the disease.

To model *in vivo* the serum bile acid overload characteristic of ICP, pregnant mice were fed with a CA-supplemented diet. Cholic acid is a primary bile acid which not only facilitates the absorption of dietary lipids and lipid-soluble vitamins in the intestine but also serves specific hormone-like functions in the control of lipid, glucose and energy metabolism. These regulatory functions of CA are mediated by signalling through bile acid receptors, particularly the farnesoid X receptor (FXR) and G-protein coupled bile acid receptor 1 (TGR5) (Thomas et al., 2008).

FXR is a member of the nuclear receptor superfamily and upon ligand activation it forms a heterodimer with RXR that binds in the promoter regions of genes controlling bile acid homeostasis and nutrient metabolism (Calkin and Tontonoz, 2012). FXR regulates the enterohepatic circulation and the bile acid synthetic cascade also indirectly by stimulating the expression of the small heterodimeric partner (SHP) protein which represses the transcription of key genes involved in the biosynthesis of bile salts (Calkin and Tontonoz, 2012). Cholic acid is an endogenous FXR agonist, however, its potency is lower when compared to other primary bile salt such as chenodeoxycholic acid and lithocholic acid (Thomas et al., 2008).

CA can also influence metabolism through signalling via TGR5, which is a member of the rhodopsin-like superfamily of G protein-coupled receptor that is highly expressed in liver Kuppfer cells and cholangiocytes, and also in gallbladder epithelial cells and immune cells (Thomas et al., 2008). Activation of TGR5 by endogenous ligands triggers internalisation of the receptor, subsequently inducing cAMP production and PKA activation. PKA phosphorylates the cAMP-response element binding protein and induces the transcription of its target genes, resulting in tissue-specific effects (e.g. gallbladder relaxation, energy expenditure in BAT and anti-inflammatory cascades) (Thomas et al., 2008).

Previous studies have shown that oral administration of cholic acid to mice increases their serum bile acid concentrations (Watanabe et al., 2006) and at the same time this treatment is compatible with pregnancy (Papacleovoulou et al., 2013). Moreover, reports in the literature confirm that chow diet supplemented with 0.5% of CA is not toxic for mice and does not affect their food intake and liver enzymes (Watanabe et al., 2006). For the purpose of our experiments, the CA feeding was initiated following the identification of the copulatory plug (marking the beginning of mouse pregnancy) in order to recapitulate more closely the phenotype of the disease in humans where cholestasis is predominantly absent outside of pregnancy. The mice were studied on day 14 of pregnancy, advanced gestation, in order to avoid possible interference of inflammatory processes mediating labour later in pregnancy. Also, unlike in humans where mammary glands are situated only in the breast, the mammary tissue in mice interweaves through the subcutaneous fat; moreover, these mammary glands branch out and expand as gestation progresses. Therefore, studying WAT during advanced and not in late murine pregnancy would minimise the interference of mammary tissue.

The results from our study confirm that feeding pregnant mice with a CA-supplemented diet for a period of 14 days is sufficient to recapitulate the changes in the serum lipid profiles detected in women with ICP: raised LDL-cholesterol, triglycerides and free fatty acids and reduced HDL-cholesterol (Martineau et al., 2015). In contrast, administration of CA to non-pregnant mice induced the same alterations in their circulating cholesterol profiles, however it did not have an effect on the fatty acid abundance and, in fact, reduced the concentrations of serum triglycerides.

Our data confirmed previous reports showing that *in vivo* administration of CA to mice increases the circulating levels of LDL-cholesterol (Watanabe et al., 2004). However, the exact mechanism underlying this effect remains unclear and therefore it could be attributed to both FXR-dependent and independent pathways. Specifically, FXR-deficient mice present with raised LDL-cholesterol levels while the transcript abundance of hepatic LDLR is not altered (Sinal et al., 2000). *In vitro* studies in cultured human cell lines have demonstrated that CDCA

stimulates an MAPK signalling cascade and elevates the mRNA level of LDLR; this process, however, does not involve the activity of FXR (Nakahara et al., 2002). On the other hand, CDCA is able to potentiate the activity of LDLR by specifically stimulating FXR and thereby inhibiting the action of proprotein convertase subtilism/kexin type 9 (PCK9) (a key regulator of cholesterol homeostasis which binds to LDLR, thereby promoting its degradation) (Langhi et al., 2008). However, there are conflicting reports in the literature regarding the impact of CA administration on LDLR activity. Specifically, *in vitro* studies have suggested that the agonistic properties of CA towards FXR are not potent enough to reduce the expression of LDLR or promote the inhibitory function of PCK9 (Langhi et al., 2008), whereas feeding of mice with a CA-supplemented diet is able to decrease the mRNA levels of LDLR in the liver (Watanabe et al., 2004). The discrepancy in these results could stem from the fact that cholic acid is a primary bile acid and as such it is biotransfomred *in vivo* to different secondary bile acid species which could act as endogenous agonists and antagonists to various receptors, including FXR. In contrast, bile acids administered *in vitro* to cultured cells do not undergo the same secondary modifications.

In humans, activation of FXR with CDCA has no effect on serum LDL-cholesterol concentrations, possibly due to the FXR-dependent inhibition of Cyp7a1 transcription resulting in reduced demand for cholesterol for bile acid synthesis and decreased LDL uptake (Wang et al., 2006). Consistent with this, deactivation of FXR, with the help of bile acid sequestrants that limit the availability of its endogenous ligands, lowers circulating LDL-cholesterol levels (Insull, 2006).

Furthermore, our results are in agreement with previous studies demonstrating that *in vivo* activation of FXR decreases HDL-cholesterol levels by raising the expression of the scavenger receptor Sr-b1 in the liver and promoting hepatic cholesterol uptake and clearance (Zhang et al., 2010b). In contrast, FXR-deficient mice present with elevated HDL-cholesterol concentrations as a consequence to the reduced expression of hepatic Sr-b1 and decreased rate of plasma HDL cholesterol ester clearance (Lambert et al., 2003).

In addition, feeding of non-pregnant mice with a CA-supplemented diet reduced the levels of their serum triglycerides and this effect is likely to be both FXR- and TGR5-dependent. The rationale behind this conclusion stems from the observations that while pharmacological activation of FXR improves plasma triglyceride clearance in wild-type lean mice by increasing the expression of ApoC2 (an LPL activator) (Kast et al., 2001) and decreasing the mRNA abundance of ApoC3 (an LPL inhibitor) (Claudel et al., 2003); this effect is lost in animals devoid of FXR (Ma et al., 2006). In addition, FXR induces the expression of the VLDL receptor and thereby facilitates the removal of triglyceride-rich VLDL particles from the blood (Sirvent

et al., 2004). It also inhibits hepatic *de novo* lipogenesis by repressing the activity of SREBP1c in a SHP-dependent manner (Watanabe et al., 2004). In humans, administration of a CDCA treatment to patients with hypertriglyceridemia reduces the levels triglycerides in their sera (Miller and Nestel, 1974). In agreement with these observations, FXR-deficient mice present with raised serum triglyceride concentrations (Lambert et al., 2003). This phenotype could results from the enhanced synthetic rate of triglyceride-rich lipoproteins previously reported in rodents devoid of FXR (Lambert et al., 2003). Moreover, lack of the same nuclear receptor in genetically-obese mice increases the circulating triglyceride levels possibly as a consequence of the decreased expression of ApoC2 and in the absence of raised VLDL secretion from the liver (Prawitt et al., 2011). In normo- and hypertriglyceridemic patients, deactivation of FXR using bile acid sequestrants results in elevated serum triglyceride levels as a consequence of enhanced VLDL production (Beil et al., 1982).

Furthermore, pharmacological activation of TGR5 in mice with diet-induced obesity decreases their plasma triglyceride levels; this effect is a consequence of the improved liver function and reduced hepatic lipid content in these animals (Thomas et al., 2009).

Our data also suggest that administration of CA to non-pregnant mice does not affect the abundance of free fatty acids in the serum. These observations are in agreement with previously published data demonstrating that feeding of wild-type lean mice with a CA-supplemented diet does not alter significantly their circulating free fatty acid concentrations (Watanabe et al., 2004). In contrast, FXR-deficient mice present with elevated circulating free fatty acid levels mainly due to their insulin resistance and increased rates of hepatic *de novo* lipogenesis (Ma et al., 2006).

As discussed in Chapter 3, non-complicated murine pregnancy is associated with significantly raised circulating triglyceride concentrations and unaltered serum free fatty acid concentrations. The results presented in this section demonstrate that administration of cholic acid to pregnant mice is able to exacerbate the changes in lipid homeostasis observed during non-complicated murine pregnancy, thereby raising the abundance of triglycerides and free fatty acids present in the blood of these rodents to supraphysiological levels. We show for the first time that the lipid metabolism of pregnant and non-pregnant mice adapts differently to a CA challenge. According to the literature, similar discrepancies have been observed in the response of lean and obese mice to changes in bile acid receptor activity. Specifically, CA-mediated activation of FXR reduces the abundance of circulating free fatty acids in high fat diet-fed rodents while this effect is absent in chow-fed controls (Watanabe et al., 2004). Also, it has been shown that pharmacological activation of TGR5 reduces plasma free fatty acid levels in diet-induced obese mice (Thomas et al., 2009).

Even though the supraphysiological increase in the serum concentrations of free fatty acids observed in pregnant CA-fed mice could be partially attributed to the downstream effects of bile-acid mediated activation of FXR and TGR5, the rise in the circulating levels of triglycerides could not be explained by alterations in the activity of either of these receptors. Since WAT plays a key role in the regulation of triglyceride and free fatty acid availability in the serum, we studied the effect of CA feeding during pregnancy on fat physiology. To acquire a more comprehensive understanding of the effects of bile acid overload on adipose tissue metabolism, subcutaneous and visceral fat, two depots shown to play contrasting role in the regulation of lipid homeostasis, were analysed in parallel.

The results from our study demonstrate for the first time that cholic acid impairs WAT adipogenesis in pregnant mice and therefore they present with smaller adipocytes and reduced triglyceride content in subcutaneous and visceral fat. Since administration of a CA-supplemented diet to non-pregnant mice for a period of 14 days did not decrease the mean adipocyte area and triglyceride content in their WAT depots we have concluded that bile feeding prevents gestation-induced lipid accumulation instead of promoting hydrolysis of existing energy stores.

Previous reports have demonstrated that delivery of CA to mice trough diet promotes triglyceride lipolysis and energy expenditure via a TGR5-dependent mechanism which activates type 2 iodothyronine deiodinase (D2) in a cAMP-dependent manner (Watanabe et al., 2006); D2 is an enzyme which facilitates the conversion of the inactive prohormone thyroxin into the active tri-iodothyronine which initiates a signalling cascade that culminates into a UCP-1 mediated thermogenesis (Bianco et al., 2002). As a consequence, CA administration is able to reverse diet-induced adiposity, decreasing white fat mass and reducing the sizes of adipocytes (Watanabe et al., 2006). Moreover, it has been concluded that CA signals these physiological adaptations via TGR5 and independently of FXR, given that administration of an FXR-specific synthetic agonist to obese mice is not able to recapitulate the effects of bile acid feeding on energy homeostasis (Watanabe et al., 2006). These conclusions have been corroborated by studies showing that treatment of obese mice with a TGR5-specific synthetic agonist is able to reverse diet-induced subcutaneous and visceral adiposity (Thomas et al., 2009). However, it could not be excluded that FXR plays an indirect role in the CA-induced changes of WAT homeostasis since FXR-deficient mice exhibit reduced subcutaneous and visceral adipose tissue mass and elevated circulating free fatty acid levels, exactly the same phenotype observed in our bile acid-fed pregnant mice. It is plausible that in pregnant mice cholic acid is metabolised so that the abundance of endogenous FXR antagonists, such as tauro-conjugated β- and α-muricholic acids (Sayin et al., 2013), is increased thereby deactivating this nuclear receptor. Moreover, previous studies in our lab have demonstrated that epiallopregnanolone sulphate is a

progesterone metabolite, supraphysiologically raised in women with ICP, which is able to competitively inhibit FXR function in the liver (Abu-Hayyeh et al., 2013). It is likely that sulphated progesterone metabolites could inhibit FXR signalling in the adipose tissue of pregnant women, thereby interfering with the physiological function of this tissue and precipitating the development of dyslipidaemia

Moreover, differences in secondary bile acid metabolism could explain the discrepancies in the response of pregnant and non-pregnant mice to CA administration. Previous studies from our lab have demonstrated that non-complicated murine pregnancy is associated with an increase in the bile acid pool (Milona et al., 2010); however, there is no record on how murine pregnancy affects bile acid species abundance and diversity. It is known, however, that gut microbial community composition and structure are profoundly altered over the course of pregnancy (Koren et al., 2012). Since the gut microbiome has a profound impact on bile acid metabolism by promoting deconjugation, dehydrogenation, and dehydroxylation of primary bile acids, it is conceivable that gestation-specific remodelling of bacterial populations in the small intestine and colon could alter the bile acid profile of pregnant women thereby altering the abundance of various bile acid receptor agonists and antagonists. Consequently, administration of the same cholic acid-enriched diet to pregnant and non-pregnant mice could induce unique downstream signalling events that result in specific adaptations in lipid metabolism.

On the other hand, it should be also acknowledged that CA administration could possibly impair adipose tissue lipogenesis also in non-pregnant mice but this phenotype may remain masked in the absence of a gestational challenge requiring rapid fat deposition (normally, adult mice undergo very gradual changes in their adiposity). To test this possibility, non-pregnant mice should be fed with a CA-supplemented diet for a period longer than 14 days. However, this experiment was outside the scope of the work presented in this document which aims to determine the contribution of bile acid overload on WAT adipose tissue function during pregnancy.

Furthermore, the results from our study demonstrate that CA administration during murine pregnancy indeed impairs subcutaneous and visceral fat lipogenesis as evidenced by the decrease in the genes expression of Pparγ2 itself as well as its targets Lpl, Ap2, Pepck and Glut4.

Although, FXR plays a key role in PPARγ-dependent adipogenic differentiation of mouse embryonic fibroblasts (Abdelkarim et al., 2010), in its absence the transcript abundance of Pparγ and its target genes in white fat is generally unaffected (Cariou et al., 2006). Since FXR-deficient mice are unable to correctly accumulate triglyceride during the course of adipocyte

differentiation and therefore exhibit decreased fat mass with a reduced adipocyte size, it has been theorised that lack of FXR inflicts an unidentified defect in triglyceride metabolism at a step distal from fatty acid uptake in adipocytes (Cariou et al., 2006). Therefore, we could exclude increased conversion of CA into secondary bile acids with antagonistic properties towards FXR as a potential mechanism leading to the diminished lipid accumulation in the adipocytes of pregnant mice fed with a bile acid-supplemented diet (Rizzo et al., 2006). Moreover, *in vivo* studies have demonstrated that pharmacological activation of FXR stimulates the mRNA expression of Pparγ2 and its metabolic targets involved in adipocyte lipid accrual. Consequently, we have concluded that CA signals the reduction in subcutaneous and visceral WAT lipogenesis in pregnant mice independently of FXR.

TGR5 also plays a role in adipose tissue lipogenesis as illustrated by the fact that mice lacking this receptor accumulate more fat (Thomas et al., 2009). Although reduced energy expenditure could be conducive to this phenotype, the exact molecular mechanisms facilitating lipid accrual in these mice have not been elucidated. Therefore, it is possible that CA could activate TGR5 in WAT which then directly or indirectly stimulates signalling cascades that impair the ability of white adipose tissue to hydrolyse and release triglycerides from lipoproteins and to internalise free fatty acids, packaging them into cytoplasmic lipid droplets.

4.4.3 Gestational cholestasis reduces adipose tissue inflammation and remodelling, thereby contributing to the development of dyslipidaemia

Administration of CA-supplemented diet to pregnant mice in order to model gestational cholestasis not only reduces subcutaneous and visceral fat lipogenesis but also alters the endocrine profile of adipose tissue, decreasing inflammation in a depot-specific manner. Specifically, we showed for the first time that in pregnant mice cholic acid reduces the protein levels of RBP4 and PAI-1 in subcutaneous WAT and also downregulates RBP4, DPP4, Fetuin A and Endocan 1 in visceral WAT.

Classically, inflammation has been viewed as the link between obesity and metabolic dysfunction, and adipose tissue is an important initiator of this response. Studies in both humans and mice have demonstrated that obesity is associated with exacerbated accumulation of immune cells in white fat. Specifically, in lean mice, approximately 10% of cells express the macrophage marker F4/80, whereas 45-60% of cells in adipose tissues of obese animals are F4/80-positive, indicating that obesity significantly alters the ratio of macrophages to adipocytes (Weisberg et al., 2003). Moreover, adipose tissue macrophages in lean animals have an alternatively-activated phenotype (M2 macrophages), have a lower inflammatory potential and are uniformly dispersed throughout the adipose tissue, whereas adipose tissue macrophages of

obese mice have a pro-inflammatory, classical phenotype (M1 macrophages) and are primarily assembled in "crown-like" structures around necrotic adipocytes (Lumeng et al., 2007). It has been proposed that adipocytes in obese subjects expand progressively in attempt to store excess dietary lipids and often become necrotic, producing chemotactic signals that recruit inflammatory monocytes, which then differentiate into classically-activated macrophages; M1 macrophages converge on necrotic adipocytes in order to facilitate their clearance (Olefsky and Glass, 2010, Chawla et al., 2011). M1 macrophages are also a major source of pro-inflammatory cytokines which can function in a paracrine and endocrine fashion to recruit more immune cells, establishing a feedforward inflammatory cascade that culminates in the development of insulin resistance (Olefsky and Glass, 2010, Chawla et al., 2011).

However, recent studies have discredited the idea that adipose tissue inflammation has a negative impact on lipid metabolism, demonstrating that pro-inflammatory signalling in WAT is in fact indispensable for heathy fat remodelling and expansion (Wernstedt Asterholm et al., 2014). Specifically, neutralisation of adipose tissue acute pro-inflammatory signalling in mice impairs their ability to accumulate visceral and subcutaneous fat mass in response to dietary challenge; consequently, these animals develop insulin resistance and metabolic dysfunction (Wernstedt Asterholm et al., 2014). It has been further demonstrated that the ability to mount an acute inflammatory response is crucial for extracellular matrix remodelling and angiogenesis (Wernstedt Asterholm et al., 2014). Although the authors of this study have not examined the impact of the neutralisation of WAT inflammatory potency on serum lipid profiles, they have demonstrated that it promotes ectopic lipid deposition in the liver. Based on these data, we have concluded that the impact of cholic acid on lipid metabolism during pregnancy is multifaceted and includes impaired adipose tissue remodelling and expansion as a consequence of reduced inflammation in this organ.

Also, it is likely that CA affects WAT inflammation and homeostasis by signalling via the TGR5 receptor. The rationale lying beneath this hypothesis is a recent study, which has convincingly demonstrated that this bile acid receptor plays a key role in adipose tissue inflammation by regulating the expression of chemokines in murine macrophages (Perino et al., 2014). Specifically, TRG5 controls the inflammatory response of immune cells residing in white fat by activating the mTOR complex 1 in an AKT-dependent manner, which in turn induces the differential translation of liver inhibitor protein, a dominant negative CEBPβ isoform (Perino et al., 2014). Therefore, chimeric mouse models which lack TGR5 in their macrophages present with exacerbated adipose tissue inflammation in response to high fat dietary challenge as evidenced by the increased abundance of crown-like structures around adipocytes and raised M1 polarisation in resident macrophages (Perino et al., 2014). Moreover, TGR5 deficiency promotes chemokine expression which then mediates obesity-induced

macrophage activation and recruitment to WAT; in agreement with this observation, pharmacological activation of TGR5 is shown to reduce the ability of macrophages to migrate *in vitro* (Perino et al., 2014). Although TGR5-dependent changes in adipose tissue inflammation are not sufficient to cause quantitative changes in fat storage (Perino et al., 2014), they could play an accessory role facilitating the decrease in white fat inflammation as a consequence of CA administration. Further studies are necessary in order to confirm that TGR5 is activated in our model of gestational cholestasis, reducing the infiltration of adipose tissue with classically-activated macrophages. Also, we could not exclude the possibility that CA reduces WAT inflammation also by affecting the expression of other bile acid receptors directly (on its own) or indirectly (via its secondary bile acid derivatives).

4.4.4 Study limitations

The most notable limitation of the work presented in this chapter is that CA-supplemented diet was used to model ICP in mice, which are otherwise not physiologically prone to developing this disease. In humans, genetic defects and/or reproductive hormones (e.g. epiallopregnanolone sulphate (Abu-Hayyeh et al., 2013)) cause impaired hepatobiliary production and excretion of bile followed by liver damage and increased excursion of bile acids into the circulation (Laatikainen and Ikonen, 1977, Williamson and Geenes, 2014). At present, there are no reports showing that ICP in humans is associated with raised bile acid concentrations in intestinal lumen and faeces. However, feeding of mice with bile acid-enriched diet increases the levels of bile in the intestine and thereby affects the enterohepatic signalling (e.g. bile acid overload in the intestinal lumen could stimulate TGR5 signalling thus promoting the secretion of glucagon-like peptide 1 from intestinal L cells which then affects insulin secretion and, whole-body glucose homeostasis (Thomas et al., 2009)). Furthermore, ICP dyslipideamia and, in particular, the derangements in cholesterol homeostasis could be largely due to the abnormal function of the liver, damaged by the intrahepatic cholestasis. In contrast, in mice fed with a CA-supplemented diet there is no evidence of hepatic dysfunction. Moreover, it is possible that the alterations in cholesterol homeostasis could stem from the excess of bile acids in the gut that leads to abnormal absorption of luminal cholesterol as a consequence of the increased emulsification of dietary fats or defective nuclear receptor signalling. However, since the work presented in this chapter focuses on the response of white adipose tissue to the bile acid overload in the serum during pregnancy, CA-feeding is able to recapitulate these conditions. On the other hand, bile acid metabolism differs between humans and mice and therefore it is likely that the primary and secondary bile acid profiles of CA-fed mice and women with ICP are qualitatively different and therefore induce distinctive signalling pathways in WAT (e.g. different bile acid species could function as agonist or antagonists for FXR).

Moreover, our results suggest that normal pregnancy and ICP have an effect on the protein expression of cytokines and adipokines in subcutaneous and visceral WAT. However, these measurements have been conducted using murine adipokine arrays, testing the enrichment of specific adipokines/cytokines in pooled adipose tissue extracts. In order to reliably confirm the changes detected with the adipokine arrays, the abundance of the same protein targets should be quantified in separate samples using enzyme-linked immunosorbent assays (ELISAs). However, the majority of ELISA kits commercially available are predominantly targeted for use on serum samples. Therefore, adapting the same assays for measurement of the enrichment of specific targets in tissue extracts requires extensive optimisation that is often unsuccessful. In future, we would attempt to adapt TNFα and RBP4 ELISA kits for the detection and quantification of these proteins in WAT tissue extracts.

Furthermore, another technical limitation of the work described in this document stemmed from the lack of specific and good-quality antibodies to evaluate the impact of normal pregnancy and ICP on macrophage infiltration in subcutaneous and visceral WAT. To address this issue, new antibodies and staining protocols will be tested and optimised for use in white adipose tissue.

A key step for the advancement of this project is the collection and comparative analysis of subcutaneous and visceral fat samples from women with non-complicated pregnancies and women with ICP undergoing elective caesarean sections; early pregnancy adipose tissue samples will be collected from women undergoing laparoscopic resections for the management of ectopic pregnancy. However, the time required for the study to be approved and authorised by the Health Research Authority and also for sufficient numbers of samples to be collected have enforced an unforeseen delay. Nevertheless, in future the results from this study would allow us to understand whether human subcutaneous and visceral WAT adapt differently to pregnancy and what molecular pathways govern the development of these changes. Moreover, we should be able to discern how ICP deregulates adipose tissue physiology and function thereby precipitating the development of gestational dyslipidaemia.

Finally, after establishing that modifications in hepatic LXR signalling together with depot-specific changes in the regulation of adipose tissue metabolism are gestational adaptations that could be conducive to the alterations in lipid homeostasis during pregnancy, we proceeded to study the impact of pregnancy on the diurnal fluctuations in key pathways regulating the energy metabolism of the mother.

Chapter 5

Impact of Pregnancy on Diurnal Metabolic Oscillations

5.1 Introduction

In mammals, major components of energy homeostasis including glucose and lipid metabolism are subjected to circadian regulation which synchronises energy intake and expenditure in accordance with predictable daily changes in the environment (Feng and Lazar, 2012). Metabolic processes are orchestrated by the master pacemaker situated in the suprachiasmatic nucleus of the hypothalamus which integrates environmental cues (e.g. light) and then entrains the peripheral clocks present in metabolic organs (e.g. liver, adipose tissue, skeletal muscle) through neuronal and hormonal signals (Feng and Lazar, 2012). The core clock machinery comprises the transcriptional activators BMAL1/CLOCK and two sets of repressors, PER/CRY and REV-ERBα and β; this molecular clock coordinates whole-body energy homeostasis by aligning the periodic expression of tissue-specific genes (Panda et al., 2002, Yang et al., 2006, Feng and Lazar, 2012).

Pregnancy is a physiologically challenging state comprising extensive adaptations in the glucose and lipid metabolism of the mother which aim to ensure a continuous supply of essential metabolites to support the growth and the development of the fetus as well as to provide the mother with sufficient energy stores to meet the demands of pregnancy and prepare for lactation (Herrera, 2002). Although previous studies have shown that gestation affects the expression of the core clock machinery in maternal organs (brain (Schrader et al., 2010, Schrader et al., 2011), liver (Wharfe et al., 2011) and placenta (Waddell et al., 2012)), there are no reports describing the impact of pregnancy on the diurnal rhythmicity of metabolic pathways in the mother. We hypothesised that the diurnal fluctuations of lipid and glucose homeostatic pathways in key metabolic tissues (liver, adipose tissue and skeletal muscle) change during early and late murine pregnancy and thereby contribute to the gestational adaptations in nutrient metabolism during these periods. Moreover, it has been reported that the placenta expresses all components of the molecular clock machinery (Ratajczak et al., 2010, Wharfe et al., 2011); however, there is a gap in our knowledge as to whether placental circadian apparatus has a functional impact on the periodic utilisation of nutrients from the maternal compartment and their subsequent transfer to the growing fetus. Therefore, we hypothesised lipid metabolism in the placenta oscillates diurnally in synchrony with circadian time.

The experimental work presented in this chapter was performed together with Dr. Georgia Papacleovoulou.

5.2 Experimental methodology

To determine the impact of pregnancy on the diurnal oscillations in the metabolic activity of mice, non-pregnant and day 14-pregnant animals were monitored in CLAMS metabolic cages for a period of 3 days. Moreover, to study the impact of pregnancy on the temporal fluctuations of lipid metabolism, mice were sacrificed on pregnancy days 7 (corresponding to early pregnancy) and 14 (representing advanced gestation). As previously described (Milona et al., 2010), non-pregnant controls were animals sacrificed one day after the identification of the copulatory plug (day 2 of pregnancy) since they were expected to be at the same stage of their menstrual cycles at that point (Figure 5.1). Non-pregnant and pregnant animals were sacrificed at 4 hour intervals over a period of 24 hours (time points corresponding to Zeitgeber 24 (ZT24; marking the beginning of the light phase), ZT4, ZT8, ZT12 (marking the beginning of the dark phase), ZT16 and ZT20). Gene expression studies and lipid profiling studies were employed to examine the impact of pregnancy on the diurnal oscillations in lipid metabolic pathways in liver, white adipose tissue, skeletal muscle and placenta.

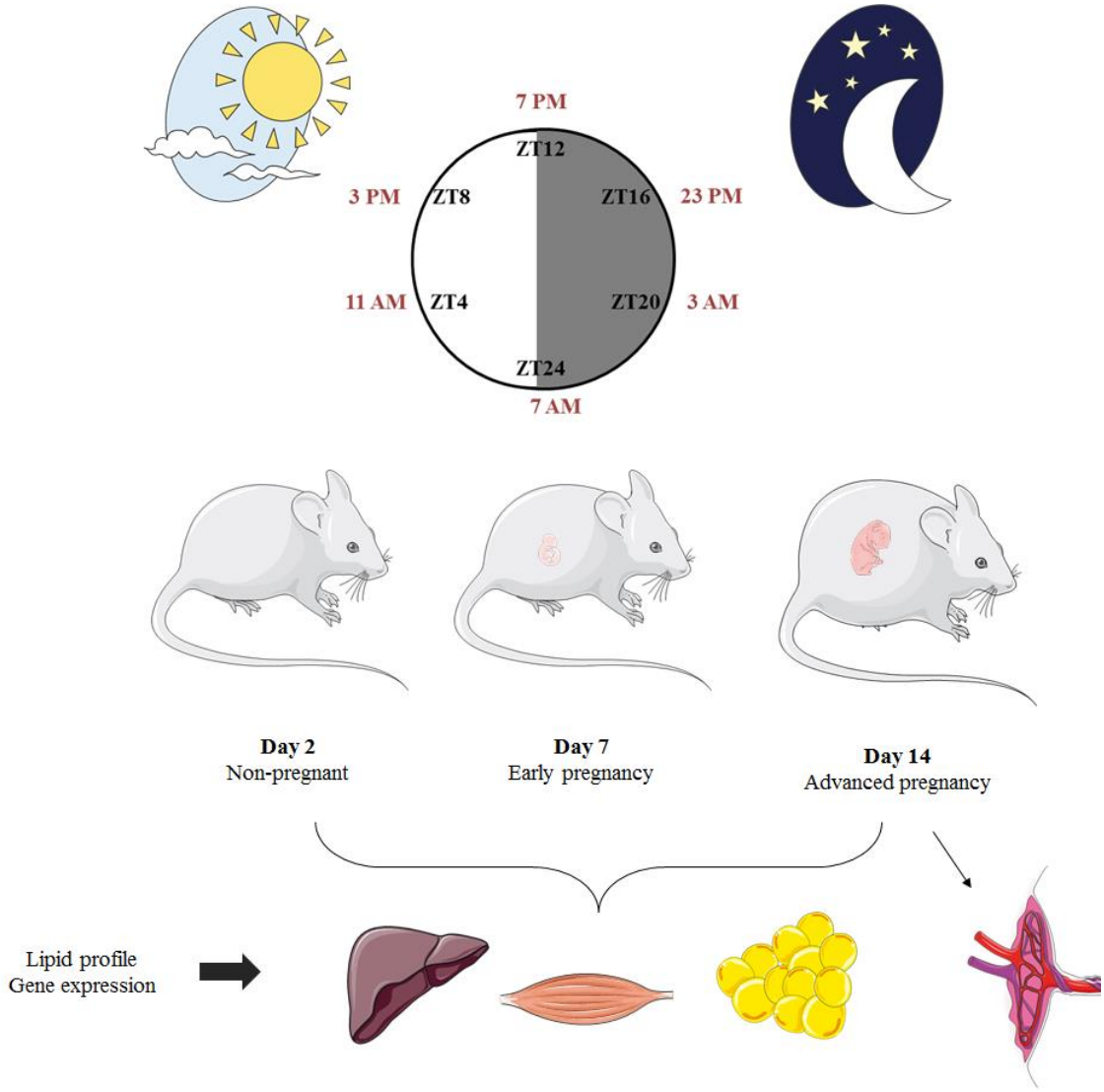


FIGURE 5.1: Schematic summary of the methodological approach employed to study the impact of pregnancy on the diurnal oscillations in lipid metabolic pathways in liver, skeletal muscle, white adipose tissue and placenta.

5.3 Results

5.3.1 Metabolic and behavioural adaptations during mouse pregnancy

We have theorised that gestational alterations in nutrient homeostasis are associated with adaptations in the diurnal rhythmicity of metabolic pathways. To interrogate this hypothesis, the metabolic and feeding activities of pregnant mice were assessed by monitoring them in CLAMS cages for a period of three days. In parallel, metabolic data were collected from age-matched non-pregnant female mice.

The results from our study indicated that there were no significant differences in the oxygen consumption, carbon dioxide release or respiratory exchange rates of pregnant and non-pregnant animals during either the light phase (7am-7pm) or the dark phase (7pm-7am) (Figure 5.2A). Although there was a trend for increased heat production by pregnant mice during both the light and the dark phases, these differences were not statistically significant.

Moreover, we compared the feeding patterns of pregnant and non-pregnant mice (Figure 5.2B and C). During the dark phase, all mice approached their cage feeders more often and also spent more time there. Pregnant mice appeared to access their feeders less than the non-pregnant mice, suggesting reduced motility. However, no differences were detected in the duration of feeding events between pregnant and non-pregnant mice. Due to technical issues we could not quantify and compare the amount of food consumed by pregnant and non-pregnant mice at different times of the day.

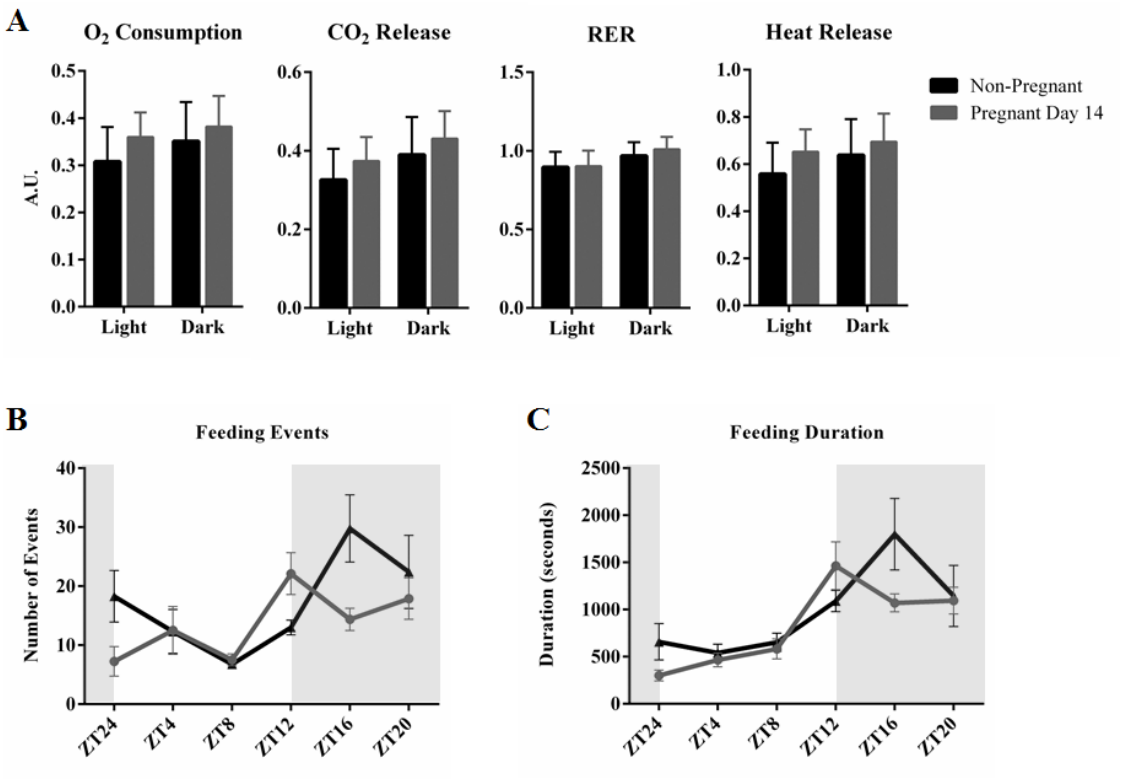


FIGURE 5.2: Metabolic and behavioural profiles of pregnant and non-pregnant mice.
Day 14-pregnant and non-pregnant mice were monitored for a period of 3 days using CLAMS cages. Grey shading marks the dark phase of the cycle. **A**. Levels of O_2 consumption, CO_2 release, respiratory exchange rate (RER) and heat release during light and dark phases. **B**. Number of feeding events per 2 hour periods (detection periods initiated at the indicated ZT time-points). **C**. Total duration of feeding per 2 hour periods (detection periods initiated at the indicated ZT time-points). Results are represented as mean ± SEM (n=6-8).

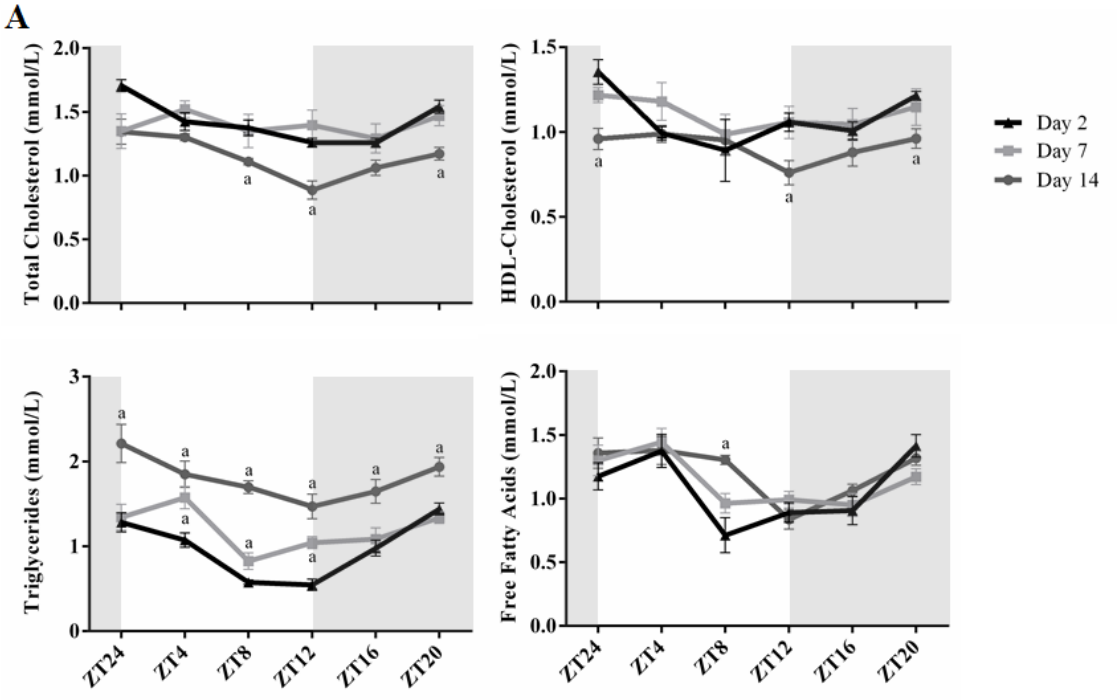
5.3.2 Impact of pregnancy on the circadian fluctuations in serum lipid biochemistry

Our previous studies have demonstrated that serum lipid profiles change at different stages of murine pregnancy (refer to Chapter 3). However, it has been established that energy metabolism adapts in response to physiological challenges not only by adjusting the total abundance of different lipid species but also by modulating their temporal distribution (Feng and Lazar, 2012). Therefore, we examined how the diurnal abundance of circulating lipids changes during early (day 7 of murine pregnancy) and advanced (day 14 of murine pregnancy) gestation as a marker of circadian metabolic function during these periods. Mice sacrificed 2 days following the identification of a copulatory plug were used as non-pregnant controls in order to minimise variations in the hormonal milieu of this experimental group.

The results from our study indicated that the diurnal concentrations of serum total cholesterol and HDL-cholesterol varied significantly over a 24 hour period in day 2 pregnant mice (Figure 5.2.2B), decreasing between ZT4 and ZT16 (Figure 5.3A); however, this rhythmicity was lost on day 7 of murine pregnancy despite the fact that no significant alterations were detected in the abundance of these two lipid species between the day 7 group and the day 2 group. On day 14 of pregnancy, the cyclicity in the diurnal variations of the serum cholesterol levels was restored and also significant differences were observed in the day 14-pregnant group when compared to the day 2 control group: serum total cholesterol levels were significantly reduced between ZT4 and ZT12 as well as at ZT20 while HDL-cholesterol levels were decreased at ZT12, ZT20 and ZT24 ($p \leq 0.05$) (Figure 5.3).

Moreover, our data showed that serum triglycerides and free fatty acid concentrations fluctuated significantly over a 24-hour period both in pregnant mice and non-pregnant controls, decreasing towards the end of the light cycle (ZT8-ZT12). However a comparison between the pregnant groups and the non-pregnant controls indicated that the levels of circulating triglycerides in day 7-pregnant mice were raised between ZT4 and ZT12, whereas in day 14-pregnant animals the abundance of these lipids was increased at all of the temporal points ($p \leq 0.05$). In contrast, the levels of free fatty acids were significantly elevated only on gestational day 14 at ZT8.

A



B

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Total Cholesterol	10.73	P < 0.0001	0.56	NS	6.92	P = 0.0001
HDL-Cholesterol	4.02	P = 0.0074	0.76	NS	1.80	P = 0.1399
Triglycerides	21.99	P < 0.0001	5.11	P = 0.0020	2.95	P = 0.0263
Free Fatty Acids	6.42	P = 0.0004	4.93	P = 0.0026	7.46	P < 0.0001

FIGURE 5.3: Diurnal variations in serum lipid profiles in non-pregnant, early pregnant and late pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase while ZT12 is at the start of the dark phase. Grey shading marks the dark phase of the cycle. Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. **A**. Serum concentrations of total cholesterol, HDL-cholesterol, triglycerides and free fatty acids. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed by one-way ANOVA with Newman-Keuls post hoc testing. **B.** Statistical analysis for rhythmic oscillations in the diurnal concentrations of serum lipids. The significance of the variations in the concentrations of serum lipids over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.3 Impact of pregnancy on the diurnal oscillations of metabolic pathways in mouse liver

Liver functions as a metabolic hub of the body which regulates nutrient and energy homeostasis (Rui, 2014). Therefore, we examined whether the circadian changes in the lipid metabolism observed during early and advanced murine pregnancy are associated with adaptations in the diurnal oscillations of key metabolic pathways in this organ.

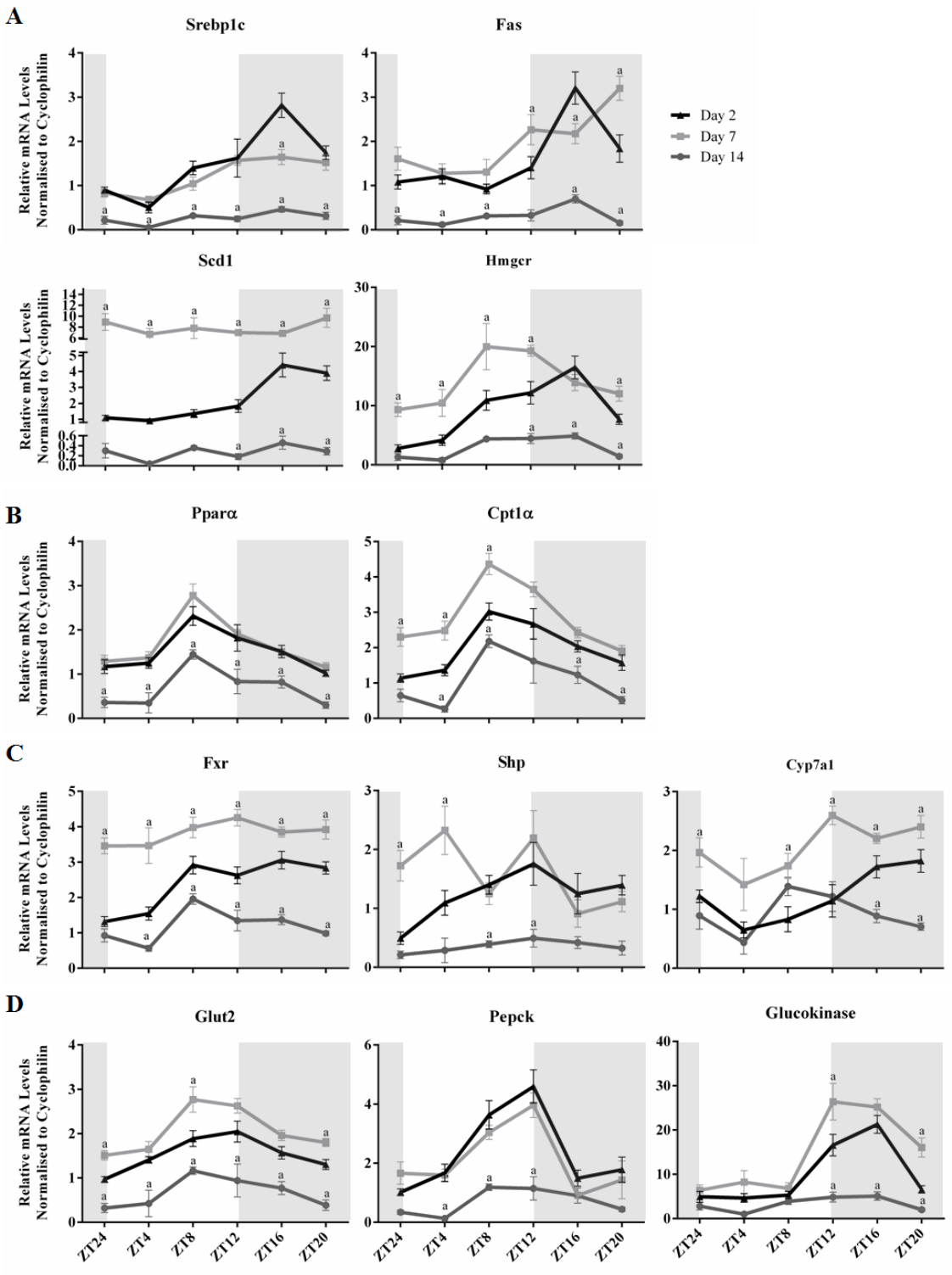
First, we studied the impact of pregnancy on the cyclicity of genes involved in hepatic *de novo* lipogenesis (Figure 5.5A). We observed that in non-pregnant animals the expression of Srebp1c, Fas, Scd1 and Hmgcr varied significantly over a 24-period (Figure 5.4E), peaking at the beginning of the dark phase, ZT16. However, the rhythmicity in the diurnal transcript distribution of Scd1 was lost on days 7 and 14 of murine pregnancy while the cyclicity and the pattern of expression of Srebp1c, Fas and Hmgcr were preserved in all pregnant animals (Figure 5.4E). Nevertheless, there were differences in the mRNA levels of these genes in pregnant mice when compared to non-pregnant controls. Specifically, the expression of Srebp1c and Fas was downregulated at ZT16 in day 7-pregnant mice ($p \leq 0.05$); however, during the same gestational period the transcript abundance of Fas was raised at ZT12 and ZT20 ($p \leq 0.05$). On the other hand, the mRNA levels of Scd1 and Hmgcr were significantly higher in day 7-pregnant mice during the majority of the 24 hour cycle than in non-pregnant controls. In contrast, the transcript abundance of Srebp1c, Fas, Scd1 and Hmgcr in day 14-pregnant mice was consistently reduced at all of the tested time points.

Next, we examined the impact of pregnancy on the circadian oscillations of genes involved in fatty acid oxidation. The expression Pparα and Cpt1α varied significantly over a period of 24 hours in both pregnant and non-pregnant mice (Figure 5.4E). However, the diurnal patterns of these two genes were similar, peaking at ZT8 in all of the experimental groups (Figure 5.4B). A comparison between pregnant mice and non-pregnant controls revealed that the mRNA levels of Cpt1α were significantly raised in day 7-pregnant mice between ZT24 and ZT8; the same experimental group did not exhibit any changes in the expression of Pparα. On the other hand, the transcript abundance of these two genes was significantly reduced on day 14 of pregnancy below non-pregnant levels.

The impact of pregnancy on the cyclicity of genes controlling bile acid homeostasis in the liver was also investigated. In non-pregnant mice, Fxr and Cyp7a1 presented with significant diurnal oscillations in their mRNA levels (Figure 5.4E): the expression of the former was raised between ZT8 and ZT20 while the latter was upregulated from ZT16 to ZT24 (Figure 5.4C). On day 7 of murine pregnancy, the expression of these two genes was increased throughout the entire 24 hour window, however Cyp7a1 preserved its cyclicity and diurnal pattern whereas the

circadian fluctuations in the transcript abundance of Fxr were lost. On gestational day 14, the expression of Fxr and Cyp7a1 presented with significant temporal variations, reaching a peak at ZT8. A comparison between day 14-pregnant and non-pregnant mice revealed that during advanced pregnancy the mRNA levels of Fxr were reduced during almost the entire 24 hour period whereas the transcript abundance of Cyp7a1 was lower at ZT16 and ZT20 ($p \leq 0.05$). Moreover, our results indicated that the fluctuations in the temporal distribution of Shp mRNA were less marked, however the expression of this gene peaked at ZT12 in the day 2 control group. On day 7 of pregnancy, the mRNA levels of Shp varied significantly, peaking at ZT4 and ZT12; this cyclicity was lost on day 14 of murine pregnancy. A comparison of the mRNA profiles of pregnant and non-pregnant mice showed that the transcript abundance of Shp was higher at ZT24 and ZT4 on day 7 of pregnancy whereas on gestational day 14, its mRNA levels were lower at ZT8 and ZT12.

Finally, we studied the impact of pregnancy on the diurnal oscillations of glucose homeostatic genes. Pregnancy did not change the rhythmicity or the expression patterns of Glut2, Pepck and glucokinse (Figure 5.4D and E). However, a comparison of the mRNA levels of these genes in pregnant mice versus non-pregnant controls revealed that on day 7 of murine pregnancy the expression of Glut2 was significantly upregulated at ZT24 and ZT8, while the transcript abundance of glucokinase was significantly elevated at ZT12 and ZT20; no changes were detected in the transcript abundance of Pepck. In contrast, the mRNA levels of Glut2, Pepck and glucokinase were significantly reduced in day 14-pregnant mice as compared to non-pregnant controls.



E

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Srebp1c	12.93	P < 0.0001	10.07	P < 0.0001	4.43	P = 0.0037
Fas	11.90	P < 0.0001	6.84	P = 0.0003	7.06	P = 0.0002
Scd1	12.43	P < 0.0001	0.85	NS	2.24	NS
Hmgcr	13.25	P < 0.0001	4.59	P = 0.0033	14.42	P < 0.0001
Pparα	7.90	P < 0.0001	15.84	P < 0.0001	7.00	P = 0.0002
Cpt1α	10.00	P < 0.0001	15.91	P < 0.0001	5.86	P = 0.0006
Fxr	12.53	P < 0.0001	1.09	NS	7.64	P < 0.0001
Shp	2.80	P = 0.0322	3.48	P = 0.0138	0.63	NS
Cyp7a1	6.28	P = 0.0003	2.97	P = 0.0277	3.72	P = 0.0094
Glut2	7.10	P = 0.0001	9.24	P < 0.0001	2.62	P = 0.0431
Pepck	12.62	P < 0.0001	10.93	P < 0.0001	4.37	P = 0.0040
Glucokinase	24.20	P < 0.0001	14.58	P < 0.0001	4.39	P = 0.0037

FIGURE 5.4: Circadian oscillations in hepatic metabolic pathways the livers of non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning of the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of key metabolic genes. **A**. Genes involved in *de novo* lipogenesis: Srebp1c, Fas, Scd1 and Hmgcr. **B**. Genes involved in fatty acid oxidation: Pparα and Cpt1α. **C**. Genes involved in bile acid homeostasis: Fxr, Shp and Cyp7a1. **D**. Genes involved in glucose homeostasis: Glut2, Pepck and Glucokinase. Results are represented as mean ± SEM (n=6-8). a $\leq$0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **E**. Statistical analysis for rhythmic oscillations in the expression of metabolic genes in the liver. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.4 Impact of pregnancy on the diurnal cyclicity of metabolic pathways in skeletal muscle

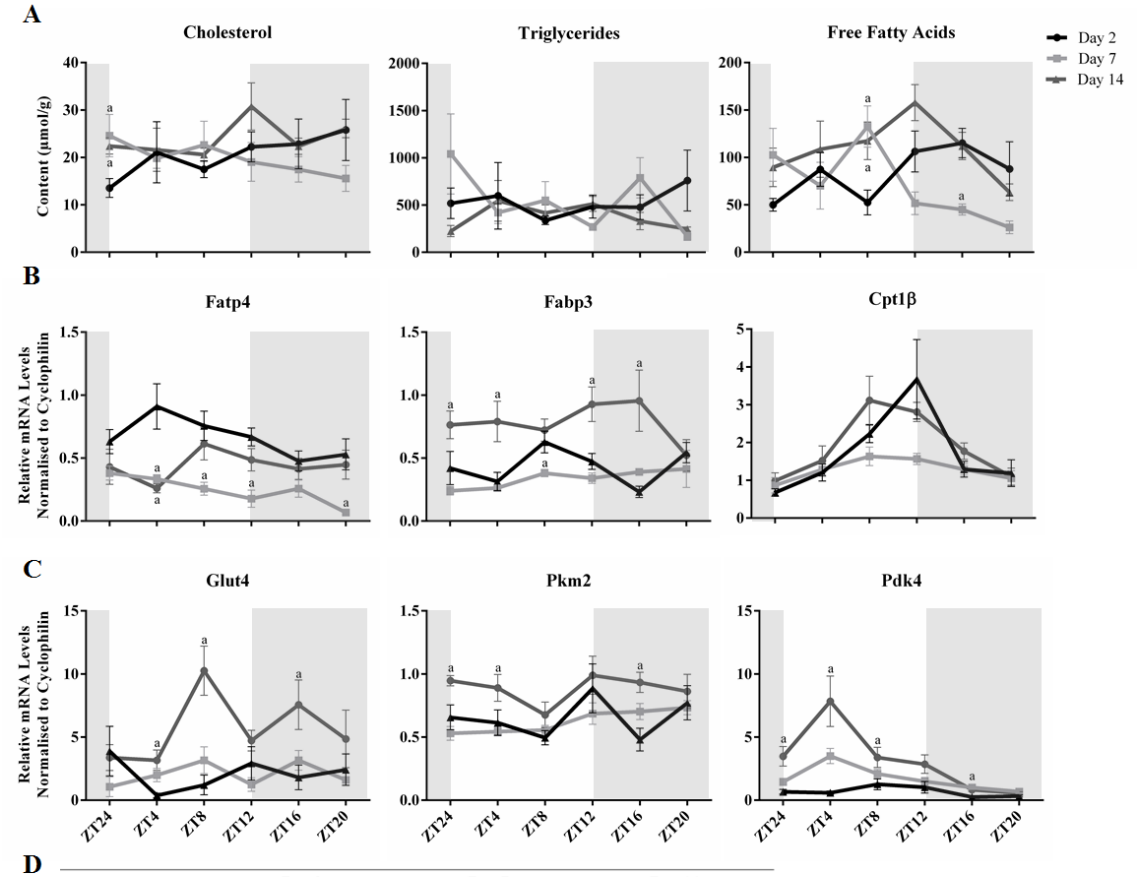
Skeletal muscle is essential for nutrient homeostasis since it determines the abundance of glucose and free fatty acids in the serum by utilising them as energy substrates to fuel its metabolic activity (Egan and Zierath, 2013). Therefore, we examined the impact of pregnancy of the circadian fluctuations of lipid metabolism in this organ in order to evaluate its contribution to the gestational adaptations in whole-body nutrient metabolism.

First, we investigated the impact of pregnancy on the diurnal oscillations of lipid homeostasis in skeletal muscle. Interestingly, the concentrations of free fatty acids in this tissue varied significantly on day 7 of pregnancy, reaching a peak at ZT8 (Figure 5.5A and D); the diurnal concentrations of this lipid species in muscle did not exhibit significant diurnal fluctuations in non-pregnant and day 14-pregnant mice. No significant differences in the abundance of cholesterol and triglycerides over a period of 24 hours were detected either in pregnant or non-pregnant mice. However, a comparison of the muscle lipid profiles of pregnant versus non-pregnant mice showed that the cholesterol content of this tissue was significantly increased at ZT24 on days 7 and 14 of pregnancy whereas the concentrations of free fatty acids were higher at ZT8 and lower at ZT16 in day 7-pregnant mice.

Furthermore, we studied how the diurnal oscillations in muscle fatty acid uptake and oxidation change on days 7 and 14 of murine pregnancy. Our data indicated that the diurnal expression of Fatp4 varied significantly only on gestational day 7 and the mRNA levels of this transporter were significantly reduced from ZT4 to ZT12 and also at ZT20 in day 7-pregnant mice as compared to non-pregnant controls (Figure 5.5B and D). The transcript abundance of Fabp3 exhibited significant temporal variations in non-pregnant animals which were lost during pregnancy. However, a comparison of the transcript abundance of this gene in pregnant mice versus non-pregnant controls showed that the mRNA levels of Fabp3 were significantly decreased at ZT12 in day 7-pregnant mice whereas in the day 14-pregnant mice, the expression of this gene was significantly raised at ZT24, ZT4, ZT12 and ZT16. Furthermore, the expression of Cpt1β presented with significant diurnal fluctuations in non-pregnant and day 14-pregnant mice; however, these fluctuations were lost on day 7 of murine pregnancy.

Finally, we examined the impact of pregnancy on the diurnal variations in the glucose homeostasis of skeletal muscle (Figure 5.5C). Although the transcript abundance of pyruvate dehydrogenase kinase 4 (Pdk4) did not present with significant temporal variations under non-pregnant conditions, its diurnal expression varied significantly on days 7 and 14 of mouse pregnancy, peaking at ZT4. Moreover, the mRNA levels of this gene were significantly raised at ZT24, ZT4 and ZT16 in day 14-pregnant mice when compared non-pregnant controls. On the

other hand, the expression of Glut2 and pyruvate kinase muscle isozyme 2 (Pkm2) did not vary significantly over a 24 hour period in non-pregnant and day-7 pregnant mice. Despite the fact the mRNA levels of these genes were raised on day 14 of mouse pregnancy as compared to non-pregnant controls, the variations in their diurnal expression did not reach statistical significance.



D

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Lipid content						
Total Cholesterol	0.90	NS	0.77	NS	1.56	NS
Triglycerides	0.46	NS	1.73	NS	1.40	NS
Free Fatty Acids	2.30	NS	3.23	P = 0.0227	2.51	NS
Metabolic genes						
Fatp4	1.74	NS	4.15	P = 0.0060	1.02	NS
Fabp3	3.07	P = 0.0220	1.49	NS	1.15	NS
Cpt1β	5.11	P = 0.0016	2.46	NS	7.04	P = 0.0001
Glut4	1.03	NS	1.66	NS	2.47	NS
Pkm2	1.74	NS	2.46	NS	0.94	NS
Pdk4	2.07	NS	8.21	P < 0.0001	9.21	P < 0.0001

FIGURE 5.5: Temporal oscillations in skeletal muscle metabolism in non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. **A**. Concentrations of cholesterol, triglycerides and free fatty acids in skeletal muscle. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. **B**. Expression of genes involved in fatty acid homeostasis in skeletal muscle: Fatp4, Fabp3 and Cpt1β. **C.** Expression of genes involved in glucose homeostasis in skeletal muscle: Glut4, Pkm2 and Pdk4. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **D**. Statistical analysis for rhythmic oscillations in the lipid

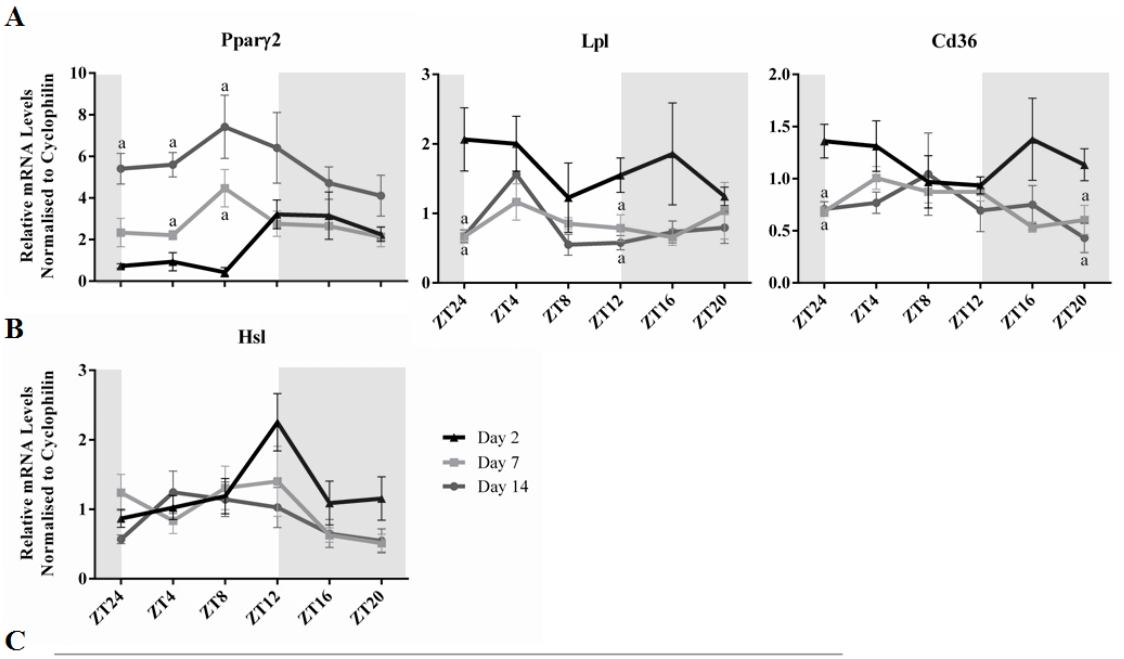
content and expression of metabolic genes in skeletal muscle. The significance of the variations in lipid concentrations and gene expression over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.5 Impact of pregnancy on the diurnal cyclicity of metabolic pathways in WAT

White adipose tissue metabolism varies prominently in the course of the day in response to the changing demands in energy homeostasis (Shostak et al., 2013a). During the active phase, nutrients taken up from diet are transported as free fatty acids or glucose to WAT where they are esterified to triglycerides and packaged in lipid droplets for storage (Shostak et al., 2013a). In contrast, during their inactive phase, mammals are under conditions of fasting and therefore triglycerides from adipose stores are released as free fatty acids to serve as energy substrates for other organs (Shostak et al., 2013b). Since WAT function is tightly controlled by circadian time, we examined the impact of pregnancy on the diurnal oscillations of metabolic pathways controlling lipid homeostasis in this tissue

First, the impact of pregnancy on adipose tissue fatty acid uptake was studied. The temporal expression of Pparγ2 exhibited significant fluctuations in non-pregnant mice, increasing between ZT12 and ZT20 (Figure 5.6A and C); this rhythmicity was lost during early and advanced murine gestation. Also, the mRNA levels of Pparγ2 were significantly raised between ZT24 and ZT8 in both day 7- and day 14-pregnant mice as compared to non-pregnant controls (Figure 5.6A). In contrast, the expression of Lpl and Cd36 in WAT was not cyclic under non-pregnant conditions. However, the differences in the diurnal expression of Lpl were significant on day 14 of pregnancy whereas the temporal changes in the mRNA levels of Cd36 reached significance on day 7 of pregnancy. On the other hand, a direct comparison of the transcript abundance of Lpl and Cd36 in pregnant and non-pregnant WAT showed the mRNA levels of the former were reduced at ZT24 and ZT12 whereas the gene expression of the latter was downregulated at ZT20 and ZT24.

Moreover, we tested whether the timing of adipose tissue lipolysis changed at the different stages of pregnancy. Our results indicated that the diurnal expression of Hsl varied significantly in non-pregnant mice, spiking at ZT12; however, this cyclicty was lost during pregnancy (Figure 5.6B and C).



C

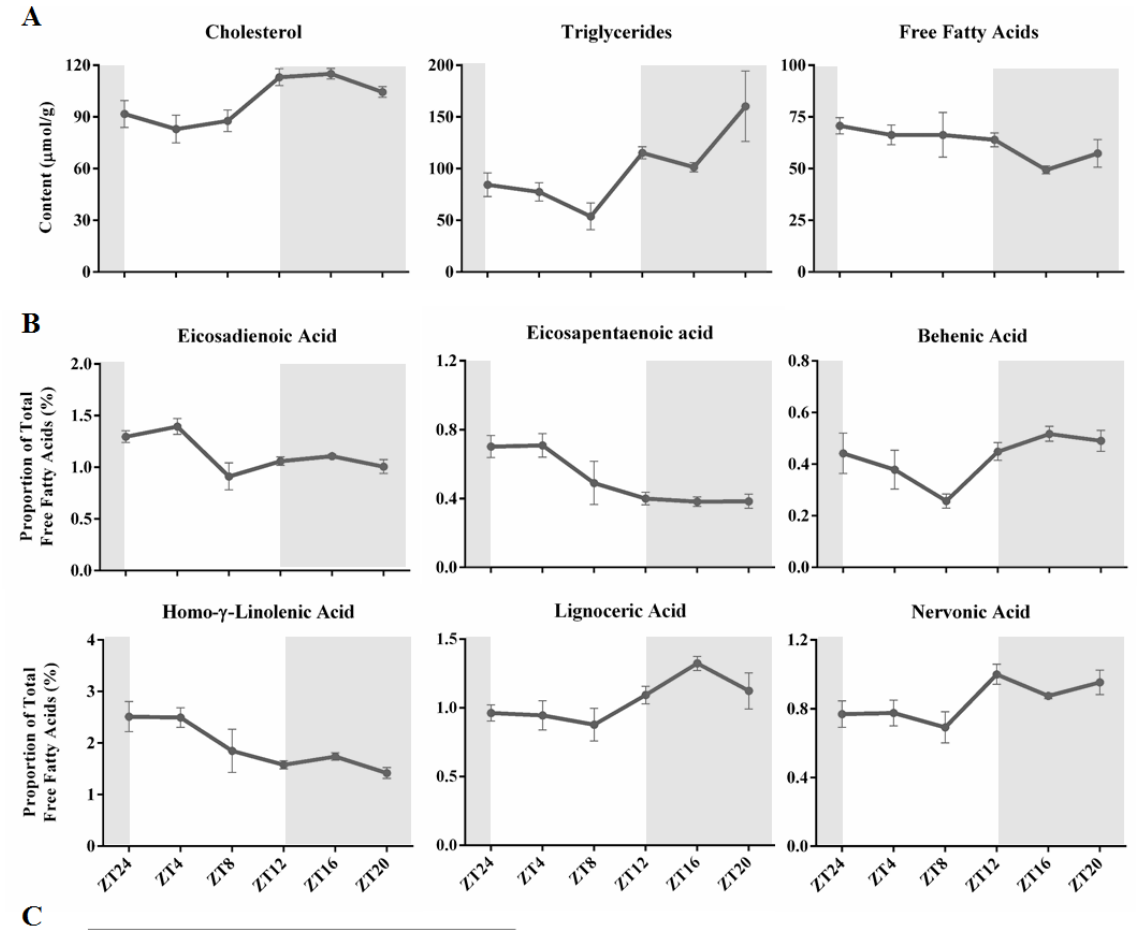
	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Pparγ2	4.19	P = 0.0053	2.27	NS	1.06	NS
Lpl	0.66	NS	0.95	NS	3.05	P = 0.0217
Cd36	0.69	NS	3.90	P = 0.0083	0.89	NS
Hsl	2.72	P = 0.0411	1.86	NS	1.90	NS

FIGURE 5.6: Diurnal oscillations in WAT metabolism in non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. **A**. Expression of Pparγ2, Lpl and Cd36 in WAT. **B**. Expression of Hsl in WAT. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **C**. Statistical analysis for rhythmic oscillations in the expression of metabolic genes in white adipose tissue. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.6 Circadian oscillations in lipid homeostasis in mouse placenta

Placenta is an organ anatomically configured to utilise metabolic substrates from the maternal compartment and then transfer them to the fetus in order to support its growth and development (Johnson, 2013). Recent studies have demonstrated that rodent placentas express key components of the core clock machinery and the expression of these genes presents with a stable diurnal oscillatory patterns (Ratajczak et al., 2010, Wharfe et al., 2011, Waddell et al., 2012). Although the authors of these reports have speculated that temporal regulation of placental physiology and function could be essential to maintain homeostatic nutrient transport to meet the demands of fetal growth, they have not presented any evidence to support their theories. Therefore, we examined whether lipid metabolic pathways in mouse placenta exhibit diurnal fluctuations.

Our data indicated that total cholesterol and triglyceride levels in mouse placenta present with a significant diurnal cyclicity (Figure 5.7C) reaching a peak between ZT12 and ZT20 (Figure 5.7A). In contrast, the total content of free fatty acids in this tissue did not exhibit any circadian fluctuations. A complete profile of the abundance of essential free fatty acid species in the placenta over a 24 hour period confirmed that 13 out of the 16 assayed molecules did not present with significant diurnal fluctuations (Figure 5.7B and Appendix II). However, the concentrations of eicosadienoic, eicosapentaenoic and homo-γ-linoleic acids showed diurnal cyclicity reaching a nadir between ZT8 and ZT20. In contrast, the levels of behenic, lignoceric and nervoic acid were raised from ZT12 to ZT20 (Figure 5.7B).



C

	Day 14	
	F value	P value
Total Lipids		
Cholesterol	5.67	P = 0.0007
Triglycerides	4.91	P = 0.0022
Free Fatty Acids	2.19	NS
Fatty Acid Species		
Eicosadienoic Acid	5.68	P = 0.0007
Eicosapentaenoic Acid	4.70	P = 0.0024
Behenic Acid	3.82	P = 0.0077
Homo-γ-Linoleic Acid	3.71	P = 0.0089
Lignoceric Acid	3.16	P = 0.0194
Nervoic Acid	3.13	P = 0.0202

FIGURE 5.7: Temporal oscillations in lipid homeostasis in mouse placenta on day 14 of pregnancy. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shanding). **A**. Concentrations of total cholesterol, triglycerides and free fatty acids in mouse placenta. **B**. Abundance of eicosadienoic, eicosapentaenoic, behenic, homo-γ-linoleic, lignoceric and nervoic acids in mouse placenta. Results are represented as mean ± SEM (n=6-8). **C.** Statistical analysis for rhythmic oscillations in the lipid content of mouse placenta. The significance of the variations in lipid concentrations over a 24-hour period was analysed using multiple measures of ANOVA.

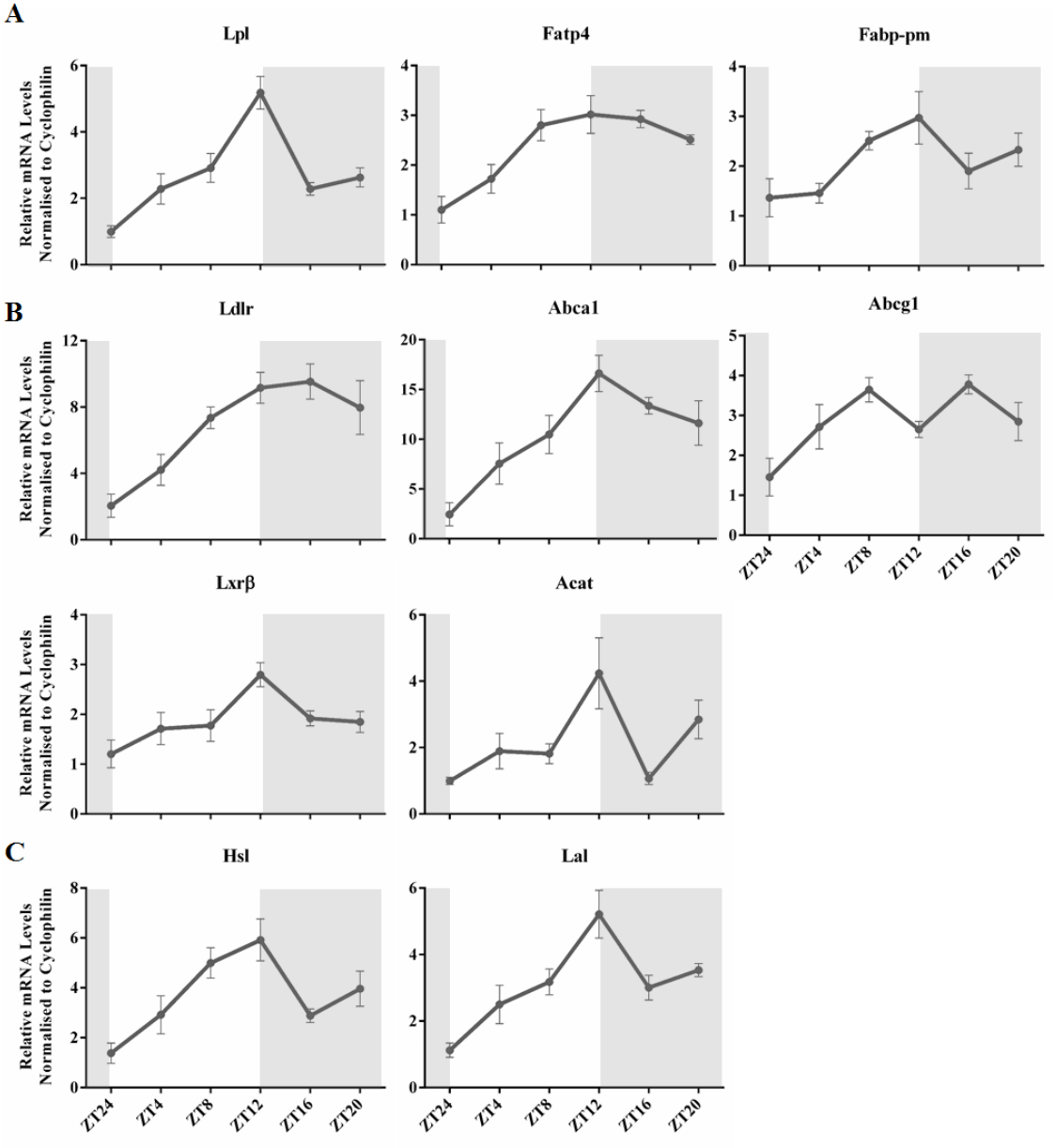
5.3.7 Circadian oscillations of lipid metabolic pathways in mouse placenta

Placental lipid homeostasis is under circadian control and therefore we aimed to determine whether molecular pathways, which have been classically associated with the utilisation of lipid molecules from the maternal circulation and their subsequent release into fetal compartment, also presented with diurnal fluctuations.

First, we examined whether placental fatty acid uptake pathways were under circadian regulation. Our results showed that the transcript abundance of Lpl, Fatp4 and Fabp-pm exhibited significant diurnal fluctuations (Figure 5.8D). Specifically, it was observed that while the mRNA levels of Lpl and Fabp-pm spiked at ZT12 and then sharply dropped at ZT16, those of Fatp4 remained raised between ZT8 and ZT20 (Figure 5.8A). Interestingly, no significant temporal fluctuation in the expression of endothelial lipase (El), Cd36 and Fatp1 were detected in mouse placenta over a 24 hour period (Appendix II).

Moreover, we studied the rhythmicity of genes regulating cholesterol homeostasis in mouse placenta. Our data indicated that expression of Abca1, Lxrβ and Acat presented with significant diurnal fluctuations (Figure 5.8D) spiking at ZT12 and then rapidly decreasing (Figure 5.8B). On the other hand, while the oscillations in the mRNA levels of Ldlr and Abcg1 were also significant, their expression patterns were different: the transcript abundance of the former was elevated between ZT8 and ZT20 whereas the expression of the latter peaked twice, at ZT8 and ZT16 (Figure 5.8B). No cyclicity was detected in the temporal expression of Lxrα in mouse placenta (Appendix II).

Since placental lipid utilisation pathways appeared to be rhythmic, we tested whether the mobilisation of lipid stores was also under circadian control. According to our results, the expression of both Hsl and lysosomal acid lipase (Lal) in mouse placenta varied significantly over a 24 hour period, spiking at ZT12 and then sharply dropping at ZT16 (Figure 5.8C and D).



D

	Day 14	
	F value	P value
Lpl	14.45	P < 0.0001
Fatp4	8.38	P < 0.0001
Fabp-pm	3.21	P = 0.0189
Ldlr	7.86	P < 0.0001
Abca1	9.08	P < 0.0001
Abcg1	4.09	P = 0.0058
Lxrβ	3.35	P = 0.0157
Acat	4.67	P = 0.0027
Hsl	6.61	P = 0.0003
Lal	9.96	P < 0.0001

FIGURE 5.8: Circadian oscillations in the expression of genes regulating lipid metabolism in mouse placenta on day 14 of pregnancy. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). RT-PCR was used to quantify the diurnal oscillations in the mRNA levels

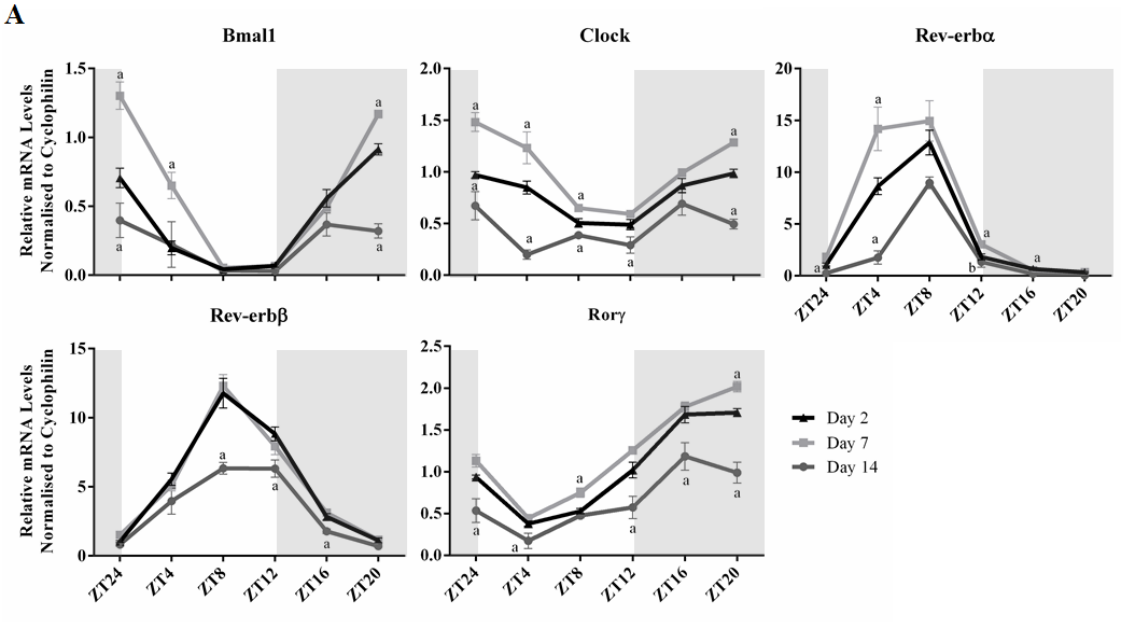
of genes of interest. **A**. Expression of placental genes involved in fatty acid uptake: Lpl, Fatp4 and Fabp-pm. **B**. Expression of placental genes in involved in cholesterol homeostasis: Ldlr, Abca1, Abcg1, Lxrβ and Acat. **C**. Expression of placental genes involved in triglyceride mobilisation: Hsl and lysosomal acid lipase (LAL). Results are represented as mean ± SEM (n=6-8). **D.** Statistical analysis for rhythmic oscillations in the expression of metabolic genes in mouse placenta. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA.

5.3.8 Impact of pregnancy on the circadian expression of clock genes in the liver

After establishing that the expression of key metabolic genes in the liver was differentially altered during early and advanced murine pregnancy, we examined whether these adaptations could be associated with changes in the activity of the hepatic core clock.

Our data indicated that pregnancy altered the diurnal activity of the hepatic clock. Although the temporal expression of the core clock genes Bmal1, Clock, Rev-erbα, Rev-erbβ and Rorγ exhibited significant rhythmicity in day 7- and day 14-pregnant mice (Figure 5.9B) and also their circadian patterns were not affected by pregnancy, there were differences in the mRNA levels of these genes in pregnant mice when compared to non-pregnant controls (Figure 5.9A). Specifically, at ZT24, ZT4 and ZT20 the mRNA abundance of Bmal1 was increased in early pregnant mice and then it was reduced on gestational day 14 ($p \leq 0.05$). Similarly, the expression of Clock was increased in the majority of the time points on day 7 of pregnancy and then reduced on day 14 ($p \leq 0.05$). On the other hand, the transcript abundance of Rev-erbα was significantly raised at ZT4 and ZT12 in early-pregnant mice and then significantly reduced between ZT24 and ZT16 in advanced-pregnant mice as compared to controls. Similarly, the mRNA levels of Rev-erbβ were significantly reduced in all circadian time points except for ZT20 on day 14 of pregnancy; the diurnal expression of this gene was not altered in early-pregnant mice as compared to controls. At the same time, the transcript abundance of Rorγ was significantly upregulated at ZT8 and ZT20 on day 7 of pregnancy and then significantly reduced during the entire 24 hour cycle in day 14-pregnant mice as compared to non-pregnant controls.

A



B

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Bmal1	60.09	P < 0.0001	76.71	P < 0.0001	3.13	P = 0.0206
Clock	18.05	P < 0.0001	20.70	P < 0.0001	5.68	P = 0.0007
Rev-erbα	71.76	P < 0.0001	31.46	P < 0.0001	87.95	P < 0.0001
Rev-erbβ	64.08	P < 0.0001	76.37	P < 0.0001	29.59	P < 0.0001
Rorγ	87.53	P < 0.0001	109.40	P < 0.0001	8.51	P < 0.0001

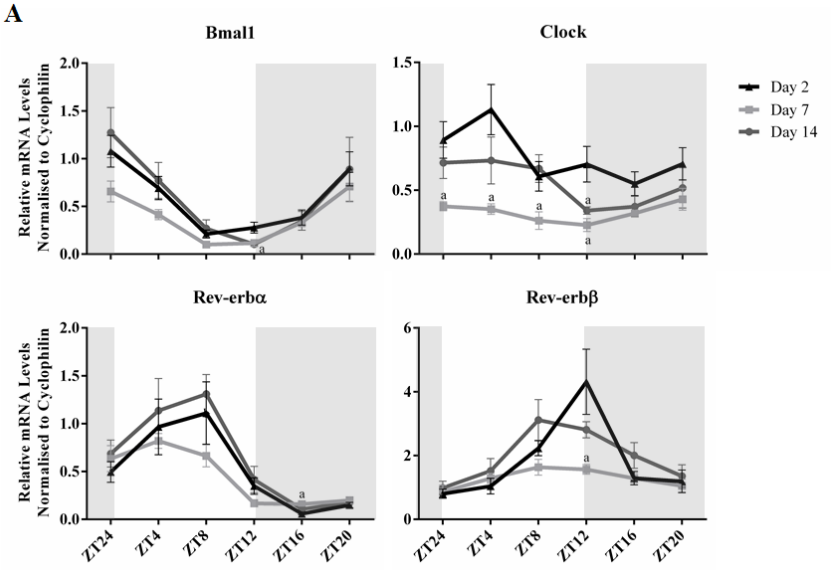
FIGURE 5.9: Diurnal variations in the expression of core clock genes in the liver of non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. Expression of Bmall, Clock, Rev-erbα, Rev-erbβ and Rorγ in the liver. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **B**. Statistical analysis for rhythmic oscillations in the expression of clock genes in the liver. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA.

5.3.9 Impact of pregnancy on the diurnal cyclicity of the core clock machinery in the muscle

In pregnant mice, the temporal expression of genes regulating the lipid and glucose metabolism of skeletal muscle was altered, therefore, we examined whether these gestational adaptations are associated with changes in the activity of the canonical pacemaker in this tissue.

The results from our study demonstrated that early pregnancy affects the circadian activity of skeletal muscle by diminishing the ciclycity Rev-erbβ (Figure 5.10B). Specifically, in non-pregnant mice the expression of this gene exhibited a significant diurnal pattern, spiking at ZT12 (Figure 5.10A). However, this peak was lost in day 7-pregnant mice and its mRNA levels were significantly reduced at ZT12 when compared to the control group. On day 14 of pregnancy, the rhythmicity of Rev-erbβ was restored but its transcript abundance peaked between ZT8. On the other hand, pregnancy had no impact on the diurnal fluctuations and the expression patterns Bmal1 and Rev-erbα. Moreover, based on our results, the expression of Clock did not present with significant temporal variations in either of the experimental groups. However, a comparison of the mRNA levels of this gene in pregnant versus non-pregnant mice showed that in day 7-pregnant mice the transcript abundance of Clock was reduced between ZT24 and ZT12 whereas in day 14-pregnant mice it was decreased at ZT12 ($p \leq 0.05$).

A



B

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Bmal1	8.05	P < 0.0001	10.06	P < 0.0001	4.33	P = 0.0039
Clock	2.40	NS	1.92	NS	1.97	NS
Rev-erbα	5.50	P = 0.0009	12.39	P < 0.0001	10.21	P < 0.0001
Rev-erbβ	8.94	P < 0.0001	2.46	NS	4.53	P = 0.0030

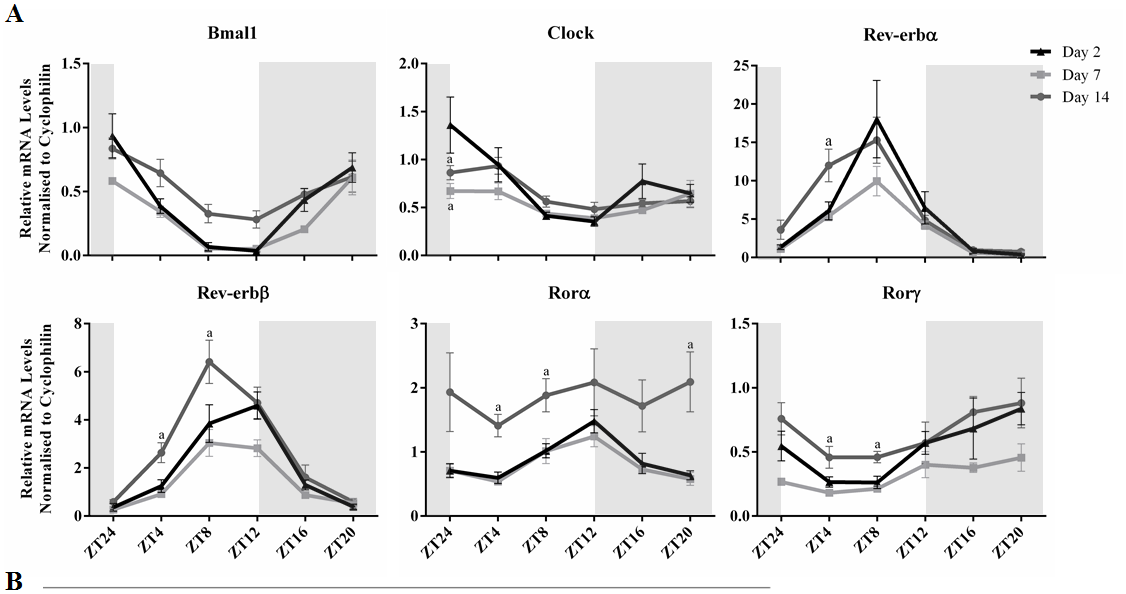
FIGURE 5.10: Circadian variations in the expression of core clock genes in the skeletal muscle of non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. Expression of Bmall, Clock, Rev-erbα and Rev-erbβ in skeletal muscle. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **B**. Statistical analysis for rhythmic oscillations in the expression of metabolic genes in the liver. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.10 Impact of pregnancy on the diurnal cyclicity of the core clock machinery in white adipose tissue

After establishing that the diurnal oscillations in the expression of lipid metabolic genes in WAT was adjusted during early and advanced murine gestation, we examined whether these gestational adaptations coincide with changes in the activity of WAT core clock during these periods.

Our results demonstrated that pregnancy had an impact on the diurnal activity of the WAT clock. Specifically, the expression of the core clock genes Bmal1, Clock, Rev-erbα and Rev-erbβ in all three of the experimental groups presented with statistically significant temporal fluctuations (Figure 5.11A and B); the expression patterns of these genes, observed under non-pregnant conditions, were also preserved during early and advanced pregnancy. However, a direct comparison of the WAT profiles of pregnant mice versus non-pregnant controls revealed that the rhythmicities of Rev-erbα and Rev-erbβ were altered on day 14 of pregnancy so that the mRNA levels of the former were increased at ZT4 while those of the latter were raised at both ZT4 and ZT8 ($p \leq 0.05$); the temporal expression of Clock was also changed by being downregulated only at ZT24 in all pregnant mice ($p \leq 0.05$). Furthermore, the cyclicity of Rorα and Rorγ was similar in non-pregnant and day 7-pregnant mice ($p \leq 0.05$). However, this rhythmicity was lost on day 14 of mouse pregnancy when the transcript abundance of Rorα was significantly raised at ZT4, ZT8 and ZT20 whereas the mRNA levels of Rorγ were significantly increased at ZT4 and ZT8.

A



B

	Day 2		Day 7		Day 14	
	F value	P value	F value	P value	F value	P value
Bmal1	13.59	P < 0.0001	22.48	P < 0.0001	5.89	P = 0.0001
Clock	5.50	P = 0.0010	3.36	P = 0.0164	7.36	P < 0.0001
Rev-erbα	8.58	P < 0.0001	17.64	P < 0.0001	14.53	P < 0.0001
Rev-erbβ	18.30	P < 0.0001	17.64	P < 0.0001	18.55	P < 0.0001
Rorα	7.20	P = 0.0002	4.94	P = 0.0022	0.28	NS
Rorγ	3.34	P = 0.0163	3.20	P = 0.0202	1.80	NS

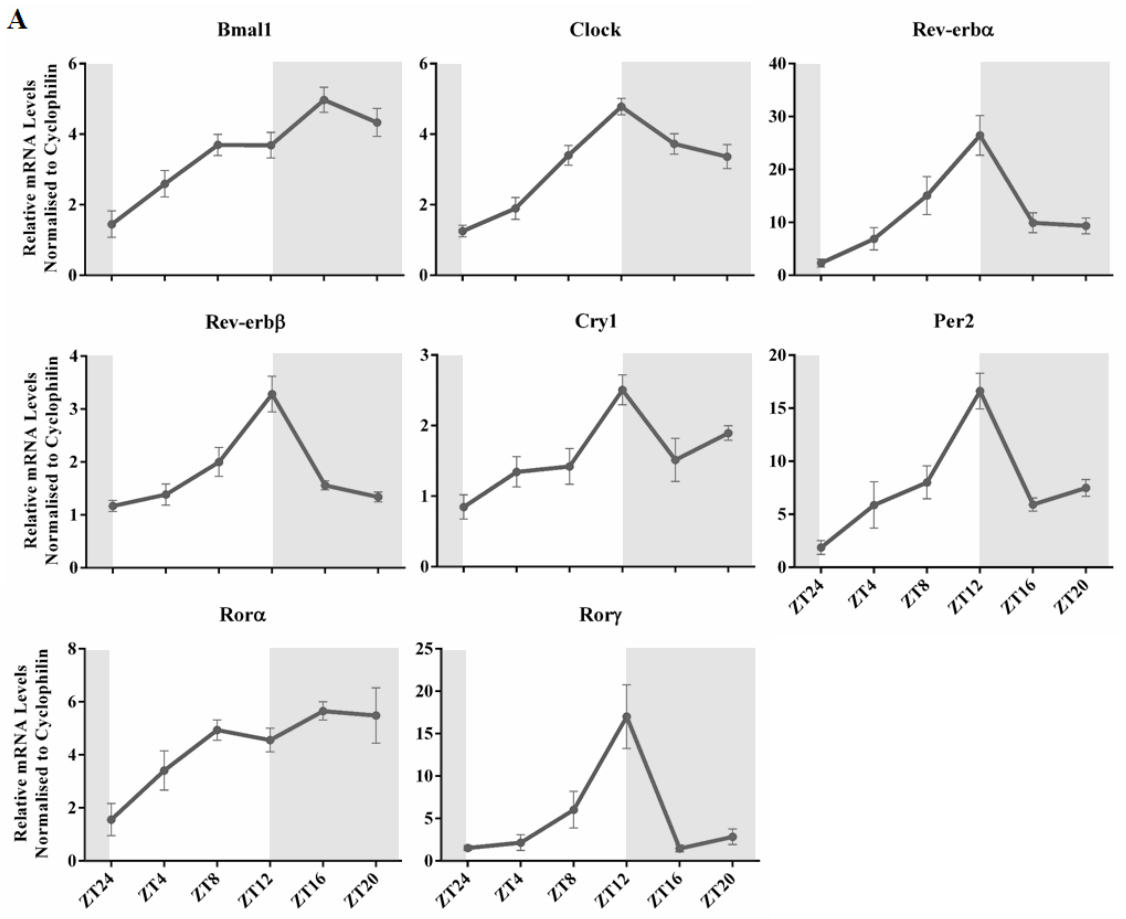
FIGURE 5.11: Temporal variations in the expression of core clock genes in the visceral white adipose tissue of non-pregnant, day 7-pregnant and day 14-pregnant mice. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 7- and day 14- pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). Female mice sacrificed on day 2 post coitum were used as non-pregnant controls. RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. Expression of Bmall, Clock, Rev-erbα, Rev-erbβ, Rorα and Rorγ in WAT. Results are represented as mean ± SEM (n=6-8). a ≤0.05 comparison day 7 and day 14 groups versus day 2 group. Data were analysed using one-way ANOVA with Newman-Keuls post hoc testing. **B**. Statistical analysis for rhythmic oscillations in the expression of clock genes in white adipose tissue. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA. NS, not statistically significant.

5.3.11 Circadian oscillations in the placental clock during mouse pregnancy

Lipid metabolic pathways in mouse placenta display robust diurnal variations on day 14 of murine pregnancy. Therefore, we examined whether these circadian fluctuations coincide temporal variations in the expression of canonical pacemakers in this organ.

Placenta is an organ anatomically configured to utilise metabolic substrates from the maternal compartment and then transfer them to the fetus in order to support its growth and development (Johnson, 2013). Recent studies have demonstrated that rodent placentas express key components of the core clock machinery and the expression of these genes presents with a stable diurnal oscillatory patterns (Ratajczak et al., 2010, Wharfe et al., 2011, Waddell et al., 2012). Although the authors of these reports have speculated that temporal regulation of placental physiology and function could be essential to maintain homeostatic nutrient transport to meet the demands of fetal growth, they have not shown any metabolic studies. Therefore, we examined whether lipid metabolic pathways in mouse placenta exhibit diurnal fluctuations.

The results from our study demonstrated that the transcript abundance of placental clock genes Clock, Rev-erbα, Rev-erbβ, Cry1, Per2 and Rorγ exhibited significant diurnal cyclicity (Figure 5.12B) and that the levels of their transcripts spiked at ZT12 (Figure 5.12A). The mRNA levels of Bmal1 and Rorα also presented with robust circadian rhythmicity ($p \leq 0.05$) increasing between ZT4 and ZT16.



B

	Day 14	
	F value	P value
Bmal1	12.19	P < 0.0001
Clock	21.11	P < 0.0001
Rev-erbα	10.71	P < 0.0001
Rev-erbβ	14.01	P < 0.0001
Cry1	6.093	P = 0.0005
Per2	12.28	P < 0.0001
Rorα	5.345	P = 0.0013
Rorγ	11.89	P < 0.0001

FIGURE 5.12: Diurnal oscillations in the expression of clock genes in mouse placenta on day 14 of pregnancy. Mice were entrained to a 12 hours light, 12 hours dark cycle. Day 14-pregnant mice were sacrificed at ZT time points 24 (0), 4, 8, 12, 16 and 20. ZT24 marks the beginning if the light phase (no shading) while ZT12 is at the start of the dark phase (grey shading). RT-PCR was used to quantify the diurnal oscillations in the mRNA levels of genes of interest. Expression of Bmal1, Clock, Rev-erbα, Rev-erbβ, Cry1, Per2, Rorα and Rorγ. Results are represented as mean ± SEM (n=6-8). **B**. Statistical analysis for rhythmic oscillations in the expression of clock genes in mouse placenta. The significance of the variations in gene expression over a 24-hour period was analysed using multiple measures of ANOVA.

5.4 Discussion

Pregnancy is the most physiologically challenging state that an organism encounters across its life cycle. It comprises complex metabolic adjustments which are essential to ensure that sufficient energy substrates are continuously supplied to fuel the development of the growing fetus while concurrently maintaining homeostasis in the mother (Herrera, 2002). Pregnancy adaptations in energy and nutrient metabolism follow a biphasic pattern: early pregnancy is mainly anabolic since it is associated with enhanced lipid deposition in maternal tissues as a consequence of enhanced insulin sensitivity (Herrera, 2002). In contrast, advanced pregnancy is an overall catabolic state involving augmented hydrolysis of stored lipids, hyperlipidaemia and insulin resistance (Herrera, 2002). Feeding, physical activity and most importantly metabolism are highly sensitive to daily environmental, nutritional and hormonal cues that in turn can program the circadian clock. Therefore, we studied whether the adaptations in energy and nutrient homeostasis that occur during early and advanced gestation are also associated with temporal adjustments in the physiology and function of the key metabolic organs. The results from this study have demonstrated for the first time that early and advanced pregnancy not only reprograms the local clocks in maternal liver, muscle and adipose tissue in a unique manner but also adjusts the timing of metabolic fluctuations in some of these organs. Moreover, we have shown that placenta is an additional metabolic organ whose capacity to utilise lipids and transfer them to the fetus is paced in accordance with circadian time.

Profiling of the diurnal fluctuations in the abundance of serum lipid at different stages of gestation demonstrated that total and HDL-cholesterol varied significantly over a period of 24 hours in non-pregnant and advanced pregnant mice, decreasing during the light phase and rising during the dark phase. Similarly, circulating triglycerides and free fatty acids presented with the same robust circadian pattern in all of the experimental groups. These changes in the availability of lipids in the serum of mice over a period of 24 hours are an accurate reflection of the diurnal habits of these animals: active and eating during the dark phase and sleeping during the light phase. During early pregnancy, the abundance of total and HDL-cholesterol in the serum was not reduced during the light phase and this is likely due to the increased biosynthesis of the sterol in the liver during this period (further discussed below).

5.4.1 Diurnal fluctuations in the metabolic activity of the liver are differentially regulated during early and advanced pregnancy

Liver is the metabolic hub of the body which integrates systemic cues (e.g. hormone signals) and governs energy homeostasis not only by modulating its own metabolic capacities but also by affecting the physiological activities of other tissues such skeletal muscle and white fat (Rui,

2014). The liver, similar to the majority of the other organs in mammals, possesses a self-sustaining molecular pacemaker which maintains homeostasis by temporally synchronising the metabolic functions of this organ (Rui, 2014).

Moreover, this study demonstrates that the diurnal fluctuations in the expression of fatty acid biosynthetic genes are uniquely adjusted during early and advanced pregnancy in order to support respectively the increase and reduction of lipogenesis during these gestational periods. Specifically, it was observed that on day 7 of mouse pregnancy the rise in the expression of Fas, which under non-pregnant conditions spiked at ZT16, was prolonged, starting from ZT12 and peaking at ZT20. On day 14 of pregnancy, the overall mRNA levels of this protein were significantly reduced throughout the entire 24-hour period and the pre-pregnancy circadian pattern was restored (sharp increase at ZT16 followed by a rapid drop at ZT20). Most striking was the change in the transcript abundance of Scd1, another key enzyme in the fatty acid biosynthetic pathway (Chu et al., 2006) whose expression was raised during the dark phase in the livers of non-pregnant mice. However, the mRNA levels of this gene were devoid of significant circadian fluctuation during pregnancy. Moreover, on gestational day 7, the expression of Scd1 was raised during the entire 24-hour window, possibly driving lipogenesis during this period, whereas on day 14 of mouse pregnancy the transcript abundance of this desaturase was diminished likely in response to the reduced necessity for *de novo* fatty acid biosynthesis. It is possible that the biphasic gestational adaptations in the mRNA levels of Fas and Scd1 could be driven by factors other than Srebp1c and Lxrα, which are classically associated with the control of promoter occupancy and expression of these two genes (Shimano et al., 1999, Schultz et al., 2000, Joseph et al., 2002, Chu et al., 2006). The reason behind this theory is the fact that although the mRNA levels of Srebp1c were reduced at all of the temporal points on day 14 of pregnancy, the expression of this transcription factor was not upregulated on day 7 of murine gestation. Also, the gene expression of Lxrα presented with no significant diurnal rhythms and overall changes in pregnant and non-pregnant mice (Appendix II).

Furthermore, our data suggested that pregnancy could also adjust the timing of cholesterol biosynthesis in the liver. This conclusion is based on the observation that the diurnal expression of Hmgcr, the rate-limiting catalyst of the mevalonate pathway in the liver (Rodwell et al., 1976), was upregulated and phase-shifted on gestational day 7, peaking at the end of the light period and not during the dark period as it did under non-pregnant conditions. This adaptation could possibly facilitate early-pregnancy lipogenesis by temporally segregating fatty acid synthesis and cholesterol synthesis thereby optimising the utilisation of lipogenic precursors during the entire 24-hour period and minimalising the competition between these two pathways for their common precursor acetyl-CoA. The transcript abundance of Hmgcr was reduced on day 14 of pregnancy and its pre-pregnancy patterns of diurnal variation (gradual peak until

ZT16 followed by a sharp drop) were restored probably in response to the loss of the lipogenic challenge. However, hepatic lipid profiling is necessary in order to confirm that the changes in the mRNA levels of Fas, Scd1 and Hmgcr during early and advanced mouse gestation have a functional impact on the overall abundance and temporal distribution of triglycerides and cholesterol in the liver.

Also, the temporal fluctuations in hepatic glucose metabolism were adjusted during mouse pregnancy. On day 7 of mouse gestation, the expression of Glut2 was increased during both the light and dark phase and the peak in the mRNA levels of glucokinase was also exacerbated. In contrast, during advanced pregnancy the transcript abundance of these two enzymes together with Pepck was reduced during the entire 24-hour period and also the amplitude of their diurnal fluctuations was diminished. Glut2 is a membrane transporter which in the fed state allows glucose to enter the cytoplasm of hepatocytes where it gets phosphorylated by glucokinase, giving rise to glucose-6-phosphate which is both a lipogenic and glycogenic precursor (Rui, 2014). During prolonged fasting, the liver utilises gluconeogenic substrates that have been either synthesised locally in the hepatocytes or which have been delivered to the liver through the circulation from extrahepatic tissues (e.g. skeletal muscle). Pepck regulates *de novo* glucose biosynthesis in the liver by catalysing the conversion of oxaloacetate to phosphoenolpyruvate (Rui, 2014). Therefore, the adaptations in the temporal expression of Glut2 and glucokinase during early murine pregnancy are likely to facilitate the enhanced deposition of energy during this period by increasing the availability of lipogenic precursors and facilitating the synthesis of fatty acids and triglycerides. In line with this theory, the dampened expression of Glut2, glucokinase and Pepck during advanced gestation in the mouse not only could contribute to the reduced lipogenic capacity of the liver but also could limit the utilisation of circulating glucose by hepatocytes thereby preserving it for transplacental delivery to the fetus. However, direct quantifications of the diurnal fluctuations in glucose and glucose-6-phosphate availability in the liver are necessary in order to confirm that the alterations in the mRNA levels of Glut2, glucokinase and Pepck have a functional impact on hepatic glucose metabolism during pregnancy.

The work presented in this document demonstrates for the first time that pregnancy changes the diurnal fluctuations of β-oxidation in the liver. PPARα is the master regulator of hepatic fatty acid oxidation and promotes this process in both the mitochondria and peroxisomes (Kersten et al., 1999), whereas CPT1α is a mitochondrial transporter which catalyses the rate-limiting step of this pathway (Nelson and Cox, 2013a). Pregnancy did not affect the timing of β-oxidation in the liver since the transcript abundance of both Pparα and Cpt1α peaked at ZT8 (when the mice were fasting) in all of the experimental groups. This result is in agreement with our observation that pregnant and non-pregnant mice have the same feeding patterns (Figure 5.2.1B) and at ZT8

(corresponding to 3:00 PM) they do not consume food. However, on day 7 of murine gestation the amplitude of the increase in the mRNA levels of Cpt1α during the light phase was significantly enhanced probably facilitating fatty acid uptake and oxidation during this period; the overall expression as well as the amplitude of the diurnal variations of both Pparα and Cpt1α were downregulated during advanced pregnancy. Further studies are necessary to elucidate the physiological significance of these changes. It could be theorised that during early pregnancy excess free fatty acids (arising from the enhanced lipogenesis in this period), which have not been converted into triglycerides, get degraded and converted into ATP to satisfy the metabolic demands of hepatocytes. During advanced pregnancy, however, β-oxidation could be dampened due to the absence of excess free fatty acids following the downregulation of lipogenesis in the liver.

Another interesting finding of this study that deserves attention is that pregnancy has an impact on the circadian regulation of bile acid homeostasis in the liver by altering the temporal pattern of bile acid synthesis. Specifically, on day 7 of pregnancy the mRNA levels of Cyp7a1, a catalyst of the rate-limiting step of the neutral bile acid synthetic pathway (Nelson and Cox, 2013c), were consistently increased during the entire 24-hour window which is in agreement with the observations the cholesterol biosynthesis and hepatic cholesterol content (Figure 3.1) were augmented at this gestational stage. It is likely that that enhanced biliary excretion of the sterol during this period is needed to protect the hepatocytes from free sterol-induced cytotoxicity. In agreement with this theory, it was observed that the transcript abundance of Cyp7a1 returned to pre-pregnancy levels on day 14 of mouse pregnancy when the mRNA expression of Hmgcr was decreased and the hepatic cholesterol content was also reduced. Moreover, our results showed that pregnancy affected not only the overall abundance of Cyp7a1 mRNA but also its temporal distribution. In particular, the peak expression of Cyp7a1 seemed to be gradually shifted during murine gestation from late dark phase in non-pregnant mice, through very early dark phase in day 7-pregnant mice, to day phase in day 14-pregnant mice. These adaptations did not seem to coincide with changes in the diurnal distribution of either Lxrα (Appendix II) or Fxr/Shp expression in mouse liver during pregnancy. Based on the literature, circadian oscillations of the enzymatic activity of Cyp7a1 is synchronous with the temporal oscillations in its mRNA levels (Kai et al., 1995) and consequently bile flow and biliary secretion of bile salt, cholesterol and phospholipids exhibit distinct daily rhythms that are consistent with the diurnal patterns of Cyp7a1 expression (Nakano et al., 1990). However, we did not observe any changes in the feeding behavior of day 14-pregnant mice which could indicate that they eat more during the light phase instead of the dark phase, thereby driving the abnormal circadian expression of the reductase. To our knowledge, this is the first report of altered bile acid homeostasis under non-pathological conditions; this phenomenon was previously described in animal models where the expression of Rev-erbα (Duez et al., 2008) or

Per1/2 (Ma et al., 2009) was genetically ablated, thus resulting in liver injury and cholestasis. Moreover, deregulated bile acid homeostasis is a common factor in metabolic diseases such as non-alcoholic fatty liver disease and Type II diabetes (Ma et al., 2006, Zhang et al., 2006), and also our group has demonstrated that mouse pregnancy is a pro-cholestatic state (Milona et al., 2010). A detailed profile of the diurnal fluctuations in the levels of bile acids in the liver and serum is necessary to determine whether the altered expression of Cyp7a1 during advanced pregnancy results in impaired bile acid production during the night.

The results from our study demonstrate for the first time that the hepatic clock is adjusted in contrasting manner during early (day 7) and advanced (day 14) mouse pregnancy: during early pregnancy the amplitude in the diurnal oscillations of the canonical clock genes Bmal1, Clock, Rev-erbα and Rorγ was increased whereas later in pregnancy, the amplitude in the temporal variations of these genes together with Rev-erbβ was reduced. These results are in agreement with previous reports showing that hepatic core clock genes continue to exhibit significant diurnal fluctuation also during pregnancy (Wharfe et al., 2011). However, the authors of the same study have also demonstrated that the mRNA levels of hepatic Clock lose their diurnal oscillations during rodent gestation (Wharfe et al., 2011) and this observation is not consistent with the findings of our study. This discrepancy is highly likely to stem from the fact the two studies have been conducted in different animal species (rat versus mouse) and during different stages of pregnancy (day 21-22 of rat gestation corresponding to late pregnancy versus day 14 of mouse gestation, corresponding to advanced pregnancy).

5.4.2 Mouse pregnancy has a limited impact on metabolic fluctuations in skeletal muscle

In mammals, skeletal muscle is the most abundant tissue since it comprises approximately 40% of total body mass (Zurlo et al., 1990). Under insulin-stimulated conditions it utilises up to 80% of glucose and thereby it plays a critical role in glycemic control and metabolic homeostasis (DeFronzo et al., 1981). Moreover, skeletal muscle is able to take up long-chain free fatty acids which, after entering the sarcoplasm, could be directly subjected to mitochondrial β-oxidation for the generation of ATP or could be diverted for esterification and storage as intramuscular triglycerides (Egan and Zierath, 2013). The metabolic activities in skeletal muscle are controlled by a local clock which could be entrained indirectly via SCN or directly via timing of feeding and exercise (Harfmann et al., 2015). Moreover, to maintain homeostasis, physiological processes in this organ are not only aligned to predictable environmental changes but also are in synchrony with the metabolic rhythms of other tissues (Harfmann et al., 2015).

An interesting novel finding of this study was the alignment in the expression of Rev-erbβ and Cpt1β in skeletal muscle during pregnancy: the peak in the transcript abundance of the

mitochondrial transporter was diminished on day 7 of pregnancy but then restored and phase-shifted to ZT8 on gestational day 14. Potential association in the expression of these two genes has been suggested only in one previous study where administration of the synthetic dual Rev-erbα/β agonist was able to enhance the expression of Cpt1β in skeletal muscle (Solt et al., 2012). Moreover, the loss in the cyclicity of Cpt1β coincided with the increase in the concentrations of free fatty acids in this metabolic tissue during early pregnancy. It could be theorised that the increased fatty acid availability in skeletal muscle during the light phase could be used to satisfy the energy demands of this organ, thereby sparing glucose for hepatic and adipose tissue lipogenesis. Nevertheless, more extensive studies are required to prove that fatty oxidation is indeed altered during early and advanced pregnancy in mice as indicated by the changes in the temporal expression of Cpt1β. Moreover, both *in vivo* and *in vitro* studies are necessary to establish whether Rev-erbβ controls expression of this transferase directly (e.g. by biding to its promoter region) or indirectly (e.g. affecting the expression of other transcription factors).

Another interesting result from this study is the observation that the expression of Pdk4, which is not rhythmic under non-pregnant conditions, exhibits significant oscillations during early and advanced murine pregnancy, spiking at ZT4. Pdk4 plays a key role in the regulation of gluconeogenesis: it phosphorylates and deactivates the pyruvate dehydrogenase complex in the muscle, thereby preventing the oxidation of pyruvate to ATP and instead preserving it as a substrate for the *de novo* synthesis of glucose in the liver (Rui, 2014). It is likely that these gestational adaptations in the diurnal expression of Pdk4 could contribute to the enhanced lipogenesis during early pregnancy and to the facilitated delivery of energy substrates to the fetoplacental unit during advanced gestation. However, further studies are necessary to show that there is increased availability of lactate in skeletal muscle during the light phase on days 7 and 14 of mouse pregnancy as a functional consequence of the increased Pdk4 activity in this tissue.

Finally, the results from our study showed that there was no significant rhythmicity in the levels of cholesterol and triglycerides in the skeletal muscles of both pregnant and non-pregnant mice. Also, the concentrations of free fatty acids in this tissue showed a high degree of similarity between the different temporal points and experimental groups. However, it is possible that more sensitive methods for quantification would discern more significant differences in diurnal availability of fatty acids in skeletal muscle. Moreover, aiming to determine whether skeletal muscle modulates the diurnal availability of free fatty acids in the serum at different stages of pregnancy by utilising these lipid molecules, we profiled the expression of different membrane fatty acid transporters (Cd36 (Appendix II), Fatp4 and Fabp3, but the obtained results were highly variable and non-consistent. Also, we attempted to determine the impact of pregnancy on

glucose homeostasis in skeletal muscle. However, the gene expression of Glut4 and Pkm2 appeared to lack a significant circadian pattern and was not altered as a consequence of pregnancy in the mouse.

Our results demonstrated for the first time that pregnancy affects the activity of the core clock in skeletal muscle by specifically altering the rhythmicity of the canonical clock component Rev-erbβ. During early pregnancy, the spike in the expression of this transcription factor, observed under non-pregnant conditions at ZT12, was lost. The diurnal fluctuations in the transcript abundance of Rev-erbβ were restored on gestational day 14, however, they were phase-shifted so that the mRNA levels of the receptor peaked at ZT8.

Interestingly, our data suggested that Clock was not rhythmically expressed in the skeletal muscle of control mice and this observation contradicts a previous report showing that the diurnal pattern of this gene exhibited a 24 hour rhythmicity (Um et al., 2011). A possible explanation for this discrepancy is the fact that although both studies used the same mouse strain, C57Bl6, the animals differed in gender. Protein quantification studies need to be performed in order to confirm that the expression of Clock does not vary diurnally in the skeletal muscle of non-pregnant female mice.

5.4.3 Mouse pregnancy has a limited impact on metabolic fluctuations in white adipose tissue

White adipose tissue metabolism exhibits significant diurnal fluctuations in response to the changing demands in energy homeostasis (Shostak et al., 2013a). During the active phase, WAT utilises dietary free fatty acids and glucose to convert them into triglycerides which are then deposited into cytoplasmic lipid droplets (Shostak et al., 2013a). During the inactive phase, WAT is under conditions of fasting and therefore stored triglycerides are hydrolysed and released as free fatty acids to serve as energy substrates for other organs (Shostak et al., 2013b). Moreover, the physiology and metabolism of WAT are stringently controlled by a self-sustaining molecular pacemaker which synchronises metabolic pathways in this tissue with feeding events and temporal changes in the energy capacities of peripheral organs (Shostak et al., 2013a).

Moreover, the pattern of expression of Pparγ2 was altered during early and advanced murine pregnancy. Under non-pregnant conditions, the mRNA levels of this nuclear receptor were consistently elevated during the dark phase thereby preparing the metabolic machinery to utilise dietary glucose and fatty acids and deposit them as fat. During pregnancy, however, the transcript abundance of Pparγ2 during the light phase was significantly elevated and as a consequence the diurnal cyclicity in the expression of this gene was lost. This adaptation could

possibly facilitate the enhanced deposition of lipids in white fat during early gestation, however, the physiological significance of the raised mRNA levels of this transcription factor during advanced pregnancy remains unclear. Protein quantification studies are needed in order to confirm whether the alterations in the transcript abundance of Pparγ2 correspond to actual changes in its peptide abundance at the different temporal and gestation stages. Such studies are even more imperative in light of the fact that the expression of the *bona fide* Pparγ2 targets Cd36 and Lpl, which are critical for the incorporation of fatty acids into WAT, did not exhibit consistent diurnal fluctuations in any of the experimental groups.

Finally, we attempted to interrogate whether the timing of lipolysis in adipose tissue changes during the different stages of pregnancy. Therefore we examined the expression of Hsl which is a key regulator of triglyceride hydrolysis and whose expression also exhibits a robust diurnal pattern (Shostak et al., 2013b). Our results demonstrated for the first time that pregnancy alters the diurnal fluctuations in the mRNA abundance of Hsl and consequently the peak in its expression, which in non-pregnant mice was observed at ZT12, was lost both on gestational days 7 and 14. However, further studies are needed in order elucidate the physiological impact of this adaptation since no significant changes were detected in the diurnal patterns of fatty acid availability in the sera of pregnant mice.

The results from our study demonstrated for the first time that the diurnal expression of the canonical clock genes Clock, Rev-erbα, Rev-erbβ, Rorα and Rorγ in WAT was minimally adjusted during advanced murine pregnancy. In contrast, early mouse gestation appeared to have no significant impact on the activity of the core clock machinery in this metabolic tissue. However, further studies are necessary to elucidate the impact of the altered core clock activity in WAT later in pregnancy on the metabolism and physiology of this tissue.

5.4.4 Lipid metabolism in mouse placenta exhibit robust diurnal fluctuations on day 14 of mouse pregnancy

Placenta is a temporary organ whose principal function is to utilise metabolic substrates from the maternal compartment and then supply them to the fetus in order to support its growth and development (Johnson, 2013). It is conceivable that metabolic pathways in this tissue need to be temporally synchronised with the feeding patterns of the mother as well as with the energy requirements of the fetus. Therefore, we examined whether lipid metabolism in mouse placenta displays a diurnal pattern during advanced pregnancy.

A key finding of this study is that lipid metabolism in the placenta varies rhythmically in response to circadian time and that metabolic pathways involved in placental lipid uptake are

controlled by the local clock and therefore peak during the dark phase. This conclusion is based on the fact that the abundance of cholesterol and triglycerides in the placenta increase consistently from ZT12 onwards and is in synchrony with the postprandial increase in availability of lipids in the serum of the mother. No significant temporal variations were detected in the levels of total free fatty acids in the placenta, however, significant diurnal fluctuations were observed in the concentrations of some fatty acids species. It is likely that fatty acids taken up by the placenta get rapidly esterified to triglycerides and therefore we were able to detect changes in the abundance of the latter and not of the former.

Moreover, our studies demonstrate that the expression of not all fatty acids transporters in mouse placenta exhibit significant temporal oscillations. Specifically, the mRNA levels of Fatp4 and Fabp-pm were raised during the dark phase whereas the transcript abundance of Fatp1 and Cd36 remained constant. Similarly, the expression of Lpl and not El presented with a diurnal pattern although both of these lipases are reported as abundantly expressed in murine placenta and with equally high capacity to hydrolyse circulating lipoprotein-bound triglycerides (Lindegaard et al., 2005). Further studies are necessary in order to detail the exact molecular pathways which mediate the placental fatty acid utilisation during the active phase in the mouse.

Furthermore, cholesterol uptake into the placenta during the dark phase is likely to be regulated by LXRβ. The expression of this nuclear receptor isoform, and not of Lxrα, presented with significant diurnal variations in this tissue, peaking at ZT12. The membrane transporters Abca1, Abcg1 and Ldlr are likely to mediate to transfer of cholesterol from the maternal circulation into the placenta since their mRNA levels rose in synchrony with the increase in the abundance of the sterol into the placenta. However, ABCA1 and ABCG1 have been implicated not only in the utilisation of cholesterol in the placenta but also in its efflux into ApoA1 and HDL receptor particles that deliver the sterol to the fetus (Stefulj et al., 2009). Since the mRNA quantification studies described in this document were performed on whole-placenta extracts, it is impossible to determine the timing of cholesterol uptake and efflux in this tissue based on the expression of these transporters in the maternal and fetal membranes of the placenta. *In vivo* studies using isotope-labelled cholesterol would be useful to examine the temporal relation between placental cholesterol utilisation and excretion.

Finally, placental fatty acid uptake and deposition appears to be concurrent with triglyceride hydrolysis since both of these pathways become activated during the night. This conclusion is based on the fact that the expression of Hsl and Lal, intracellular lipases that mediate the utilisation of stored triglycerides, peaked at the start of the dark phase suggesting that non-esterified fatty acids get liberated during this period in preparation for their efflux into the fetal

compartment. However, *in vivo* studies using isotope-labelled fatty acids would be useful to examine the diurnal variations in lipid utilisation and efflux in mouse placenta.

The work presented in this document shows for the first time a comprehensive profile of the diurnal fluctuations in the expression of canonical clock genes in mouse placenta on day 14 of pregnancy. It was observed that the mRNA levels of Clock, Rev-erbα, Rev-erbβ, Cry1, Per2 and Rorγ fluctuated robustly over a 24 hour period, spiking at ZT12; the expression of Bmal1 and Rorα also exhibited significant diurnal fluctuation and the rise in their transcript abundances were maintained between ZT8 and ZT20. This specific pattern of expression could probably ensure the activation of the placental core clock machinery and metabolism during the dark phase when nutrients are available in maternal circulation. Our results are in agreement with previous reports demonstrating that the expression of Clock, Bmal1, Cry1/2 and Per1/2 varied diurnally in the placenta during late mouse pregnancy (Ratajczak et al., 2010). Moreover, it was observed that the expression of Clock and Bmal1 peaks in parallel with Rev-erbα, Rev-erbβ, and Per2 although it has been canonically described that the diurnal fluctuations in the mRNA levels of these genes follow an anti-phase pattern since BMAL/CLOCK induces the expression of Rev-erbα, Rev-erbβ, and Per2 (Feng and Lazar, 2012). These data confirm previously proposed theory that either the transcriptional-translational interactions between clock components in the placenta differ fundamentally from those in other tissues or that the canonical feedback loops regulating the molecular clock machinery have not been fully developed in this organ (Waddell et al., 2012). Detailed studies of the changes in the promoter occupancy of clock genes in the placenta over a 24 hour period are necessary to define the molecular interactions which determine the temporal pattern of expression of clock components in this tissue.

In conclusion, the work presented in this document demonstrate for the first time that changes in the circadian clock could contribute to the gestational adaptations in lipid metabolism during pregnancy (summarised in Figure 5.13). Early and advanced pregnancy alter in a unique manner the activity of the hepatic clock and the diurnal fluctuations of key metabolic genes in the liver in order to enhance or dampen lipogenesis respectively during these gestational periods. Also, we introduced preliminary data suggesting that the temporal oscillations in bile acid metabolism could be shifted during pregnancy independently of feeding patterns. Moreover, we showed that fatty acid homeostasis in skeletal muscle is changed during early pregnancy possibly as a consequence of the REV-ERBβ-dependent downregulation of Cpt1β-mediated lipid oxidation. We presented also evidence that the molecular clock of white adipose tissue is adjusted during late pregnancy in the mouse. However, further studies are necessary in order to elucidate the physiological effect of this adaptation. Finally, we convincingly demonstrated that placental lipid homeostasis exhibits circadian fluctuations and therefore pathways mediating fatty acid

and cholesterol transport as well as triglyceride hydrolysis become activated during the dark phase, in synchrony with the activation of the local clock at this time.

5.4.5 Study limitations

A key limitation of this study was imposed by the lack of specific antibodies for the detection of Rev-erbα and Rev-erbβ. Consequently, we were unable to confirm whether the changes in the mRNA levels of these clock components observed in liver and muscle reflect functional changes in their protein availability. In future, the impact of pregnancy on the protein enrichment of these circadian regulators will be re-evaluated as soon as high-quality antibodies become commercially available.

Moreover, having demonstrated that lipid metabolic pathways in murine placenta present with robust diurnal variations, it was essential to determine whether lipid homeostasis in human placenta also exhibits circadian fluctuations. For this purpose, early pregnancy chorionic villus samples and placentas delivered during elective caesarean sections had to be collected at different times of the day and then analysed. However, due to the intricacy of the study design, the accumulation of necessary samples proved to be more time-consuming than initially anticipated; this study will be completed as soon as the predetermined sample numbers are achieved.

Furthermore, due to the lack of appropriate facilities we were unable to evaluate the impact of light-dark cycle disturbances (e.g. maintenance of mice in constant light/ darkness or swapping of light dark phases) on pregnancy metabolism. These studies would have allowed us to determine whether disrupted circadian rhythmicity could interfere with the ability of maternal physiology to adapt to the metabolic demands of pregnancy and thereby precipitate the development of gestational metabolic diseases such as obesity, GDM and pregnancy-induced hypertension. Moreover, these studies would confirm whether circadian challenges such as shift-working and travelling across different time zones during pregnancy could compromise fetal growth and development by deregulating the transport of nutrients across the placenta. Another key aspect of this study would have been to determine whether fetuses which experience circadian disturbances *in utero* get predisposed to metabolic dysfunction syndromes in adulthood. Also, it would be interesting to test whether children whose mothers have been exposed to circadian disturbances during pregnancy (e.g. nurses and flight attendants) have increased risk of developing cardiometabolic diseases later in life. It is vitally important to conduct these experiments in near future since they would allow us to gain a more detailed understanding of how reproductive hormones are able to “re-set” the circadian clock and adapt maternal lipid homeostasis in response to the metabolic demands of the fetus. Knowledge of the

molecular pathways that orchestrate nutrient metabolism during non-complicated and pathological pregnancies would facilitate the development of novel therapeutic approaches to ensure optimal fetal development *in utero* as well as metabolic fitness of the offspring later in life.

Overall, all the novel data described in this document confirm our initial hypothesis that pregnancy prepares the maternal body for the nutritional demands of the growing fetus by simultaneously modifying different systems, involved in metabolic regulation (namely cholesterol sensing and LXR signalling, depot-specific white adipose tissue metabolism and the circadian fluctuations of lipid homeostatic pathways in liver and placenta). It is thereby ensured that these otherwise distinctive pathways function in a collaborative manner so that in early pregnancy they promote lipogenesis and then during advanced gestation they ensure the continuous supply of stored nutrients to the fetoplacental unit.

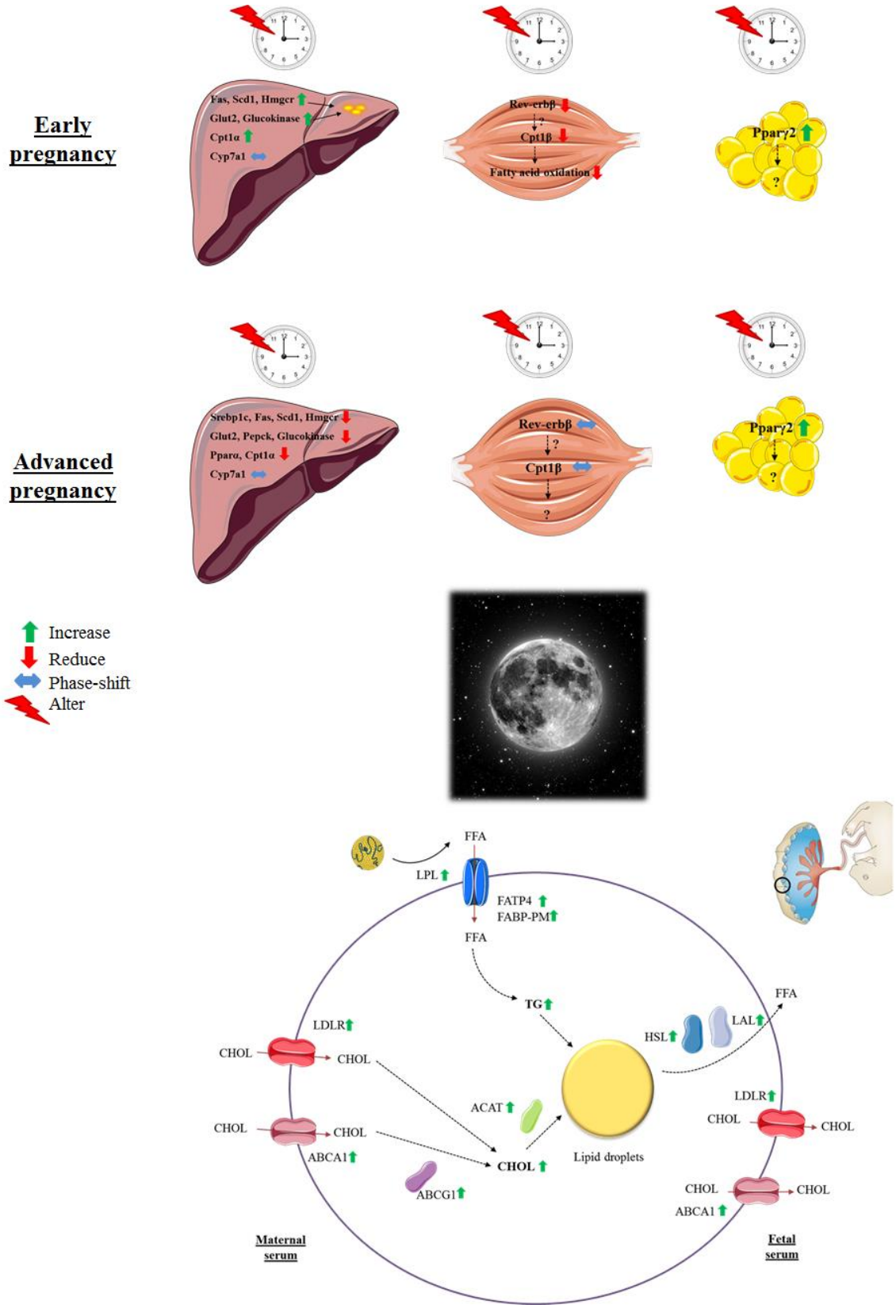
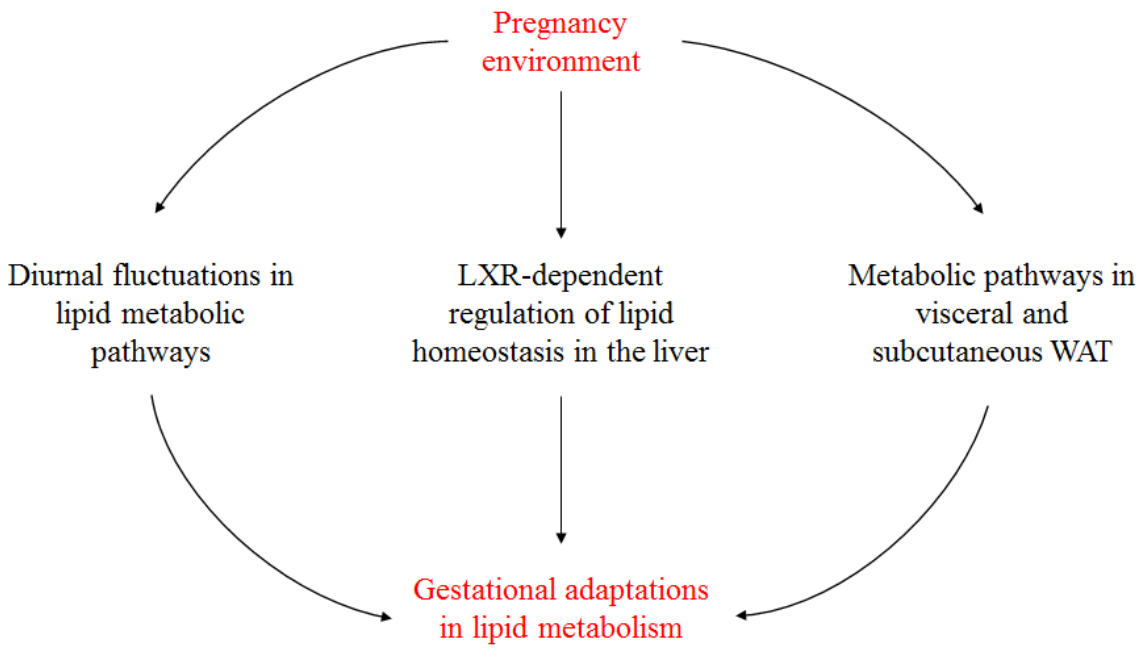


FIGURE 5.13: Summary of the impact of pregnancy on the diurnal oscillations of lipid metabolic pathways in liver, skeletal muscle, white adipose tissue and placenta.

Chapter 6

Summary of Conclusions



Pregnancy is a dynamic state involving profound changes in the maternal hormonal milieu which then signal a myriad of extensive adaptations in the lipid metabolism of the mother which aim to ensure a continuous supply of essential metabolites to support the growth and the development of the fetus as well as to provide the mother with sufficient energy stores to meet the demands of gestation and prepare for lactation (Herrera, 2002). Pregnancy adaptations in energy and nutrient metabolism follow a biphasic pattern: early pregnancy is mainly anabolic since it is associated with increased lipogenesis and enhanced lipid deposition in maternal tissues as a consequence of enhanced insulin sensitivity (Herrera, 2002). In contrast, advanced pregnancy is an overall catabolic state involving augmented hydrolysis of stored lipids, hyperlipidaemia and insulin resistance (Herrera, 2002). Although, there are a few studies which have examined the molecular factors whose activity changes during pregnancy and thereby contribute to the gestational adaptations in lipid metabolism (e.g. LPL, HSL (Alvarez et al., 1996) and glycerol kinase (Thenen and Mayer, 1975)), overall there is a sizeable gap in our understanding of the molecular mechanisms which regulate lipid homeostasis in pregnancy. Such knowledge is crucially important as evidenced by the numerous reports demonstrating that failure to maintain the levels of circulating lipids and lipoproteins within physiological ranges during pregnancy results in maternal dyslipidaemia and fetal disease (Neitzke et al., 2011, King, 2006, Yessoufou and Moutairou, 2011). Therefore, the aim of the work presented in this document was to identify additional signalling pathways which cooperatively contribute to the increase in lipogenesis in early pregnancy and the development of hyperlipidaemia during advanced gestation.

Our results provided evidence that hepatic LXR plays an important role in the regulation lipid metabolism during pregnancy. We showed that the activity of this nuclear receptor is reduced during advanced murine gestation and this could be an essential adaptation to minimalize the loss of the sterol and preserve it for an LDL-dependent delivery to the fetoplacental unit. Although, we were not able to discern the exact molecular mechanism which causes the downregulation of LXR activity in the liver we managed to obtain some preliminary data suggesting that changes in the abundance of the transcriptional coactivators Pgc1α and Pgc1β in this tissue could be responsible for the reduction in the mRNA expression of LXR target genes in advanced-to-late pregnancy in the mouse. Moreover, we presented evidence that the gestational adaptations in cholesterol homeostasis in the mouse are not associated with changes in the intestinal absorption of the sterol or its influx or efflux from peripheral tissues such as blood monocytes. Due to the physiological limitations of our experimental model, we were not able to identify the molecular mechanism which could limit the internalisation of the exogenous cholesterol which circulates abundantly throughout human gestation. To circumvent this limitation, in future peripheral blood monocytes isolated from pregnant and non-pregnant women will be studied in order to establish how extrahepatic LXR signalling changes under conditions of gestational hypercholesterolemia. Moreover, mice devoid of Lxrα/β will be examined in order to conclusively determine whether pregnancy environment signals via LXR to promote changes in key metabolic pathways that orchestrate the gestational adaptations in lipid metabolism. Detailed knowledge of the role of LXR in the regulation of lipid homeostasis throughout gestation could allow for the use of isoform-specific agonists or antagonists in order to manage respectively gestational hypercholesterolemia and hypocholesterolaemia and thereby reduce the risk of fetal atherosclerosis and microcephaly.

Moreover, we presented exciting evidence that changes in the diurnal fluctuations of metabolic pathways in mouse tissues also contribute to the changes in lipid and glucose homeostasis at different gestational stages. In agreement with this, we observed that pregnancy alters not only in the absolute levels of maternal lipids but also their diurnal fluctuations. We showed that metabolic adaptations during pregnancy are associated with significant alterations in the activity of core clocks in liver, skeletal muscle and white adipose tissue at different gestational stages. We presented evidence that early and advanced pregnancy alter in a unique manner the diurnal fluctuations of key metabolic genes in the liver in order to enhance or dampen lipogenesis respectively during these gestational periods. Also, we introduced preliminary data suggesting that the temporal oscillations in bile acid metabolism could be shifted during pregnancy independently of feeding patterns. Moreover, we showed preliminary evidence that fatty acid homeostasis in skeletal muscle could be changed during early pregnancy possibly as a consequence of the REV-ERBβ-dependent downregulation of Cpt1β-mediated lipid oxidation. We presented also evidence that the molecular clock of white adipose tissue is adjusted during

late pregnancy in the mouse, although, were not able to elucidate the physiological effect of this adaptation. Finally, we convincingly demonstrated that placental lipid homeostasis is under circadian control and therefore pathways mediating fatty acid and cholesterol transport as well as triglyceride hydrolysis become activated during the dark phase, in synchrony with the activation of the local clock at this time. In future, human chorionic villus samples and placentas delivered during elective caesarean sections will be collected at different times of the day and examined in order to determine whether human placental lipid homeostasis exhibits diurnal fluctuations. Molecular biology studies will be employed to elucidate how core clock components interact with each other forming an intricate network that tightly regulates the energy metabolism of the placenta. Moreover, a key step of our study will be examine the impact of gestational metabolic diseases such as intrauterine growth restriction not only on the placental clock but also on the circadian rhythmicity of nutrient transfer pathways in this tissue. The knowledge gained from these studies could be invaluable in designing novel therapies which target the placental clock for the prevention and treatment of abnormal fetal growth syndromes, thereby reducing the risks not only of fetal complication (e.g. preterm delivery and stillbirth) but also of adult cardiometabolic diseases.

Furthermore, we demonstrated that subcutaneous and visceral WAT function differentially in the context of physiological pregnancy and gestational cholestasis. We presented preliminary evidence that subcutaneous and visceral white adipose tissues are regulated in a contrasting manner during mouse pregnancy and therefore they could have differential contributions to lipid homeostasis during this period. Our results demonstrated that although both of these depots expand in the course of gestation in order accommodate triglyceride accrual, there is increased inflammation specifically in subcutaneous WAT whereas visceral fat remains quiescent. However, further studies are necessary to elucidate the physiological relevance of these adaptations. Also, we showed that gestational cholestasis precipitates the development of dyslipidaemia by compromising the metabolic activity of white adipose tissue. It was demonstrated that bile acid overload reduces white fat inflammation in a depot-specific manner and interferes with WAT remodelling and expansion, thereby compromising the ability of this tissue grow and store surplus lipids that normally accumulate during pregnancy. In future, subcutaneous and visceral WAT samples from women with ICP and control women with non-complicated pregnancies will be studied to determine whether bile acid overload during pregnancy interferes with gestational lipid accrual also in humans by downregulating Pparγ signalling and local tissue inflammation. Detailed knowledge of the molecular pathways dysregulated by gestational hypercholaenemia is necessary for the development of successful therapies which will target adipose tissue in a depot-specific manner in order to prevent the supraphysiological increase in serum triglycerides and free fatty acids and thereby reduce the risk of developing metabolic dysfunction later in life both in the mother and her offspring.

Overall, the work presented in this document illustrates how complex and multifaceted are the gestational adaptations in lipid metabolism and accentuates the necessity for further research in order to discern the key molecular pathways which govern these changes at the different stages of pregnancy. The data described in this study support the initially postulated hypothesis that several metabolic pathways change during pregnancy (specifically, alterations in hepatic LXR signalling, depot-specific modifications in subcutaneous and visceral WAT metabolism and adaptations in the circadian rhythmicity of nutrient and energy homeostatic pathways in key metabolic organs in the mother) and thereby cooperatively orchestrate the gestational adaptations in maternal lipid homeostasis. Moreover, it was demonstrated that bile acid overload during pregnancy interferes with white adipose tissue remodelling and expansion and thereby precipitates the development of hyperlipidaemia in murine models of gestational cholestasis. To further advance this study by integrating the knowledge derived from all three aspects of this project, it would be interesting to study whether increased serum bile acid levels in ICP could deregulate the placental clock and thereby interfere with the diurnal fluctuations of placental pathways involved in the utilisation of lipids and their subsequent transfer into the fetal compartment. It is possible that by desynchronising placental nutrient transport pathways, bile acids could also play a role in the *in utero* programing of metabolic disease previously described in the offspring from pregnancies affected with cholestasis (Papacleovoulou et al., 2013). Moreover, to further dissect the origins of supraphysiologically raised total- and LDL-cholesterol concentrations in the sera of women with ICP, it would be interesting to study whether intestinal LXR signalling is compromised as part of the pathophysiology of gestational cholestasis and therefore this nuclear receptor is not able to moderate the absorption of luminal cholesterol in the duodenum either by directly regulating the expression of cholesterol transporters (such as ABCG5/8 (Repa et al., 2002) and NPC1L1(Duval et al., 2006)) or indirectly by controlling the hydrophobicity of the bile acid pool (Peet et al., 1998). Furthermore, *in vitro* and *in vivo* studies could be conducted in order to determine whether the increased bile acid levels in ICP are able to interfere with the expression and activity of LXR in extrahepatic tissues (such as macrophages) and thus compromise the efflux of cholesterol into HDL particles and its transport back to the liver for biliary clearance; these studies would help elucidate the molecular mechanisms underlying the reduction in HDL-cholesterol levels observed in gestational cholestasis (Martineau et al., 2015). Finally, it has been established that both LXRα and LXRβ possess potent anti-inflammatory activities and upon activation they inhibit the expression of inflammatory mediators such as interleukin 6, inducible nitric oxide synthase and cyclooxygenase (Joseph et al., 2003). Also, it has been show that pharmacological activation of LXR affects fat distribution and WAT inflammation in a depot-specific manner (Archer et al., 2013). Consequently, in future it would be interesting to determine whether the bile acid overload observed in gestational cholestasis could affect LXR signalling in adipose tissue and thereby reduce subcutaneous and visceral WAT lipid accumulation and pro-

inflammatory signalling. In conclusion, detailed knowledge of the molecular mechanisms that signal in a collaborative manner to influence the adaptations in lipid homeostasis during non-complicated pregnancy, together with a thorough understanding of the molecular pathways involved in the pathophysiology of ICP, would facilitate the development of targeted therapeutic interventions not only applicable for the treatment of the dyslipidaemic phenotype of this disease, but also suitable for the management of other metabolic diseases of pregnancy such as GDM.

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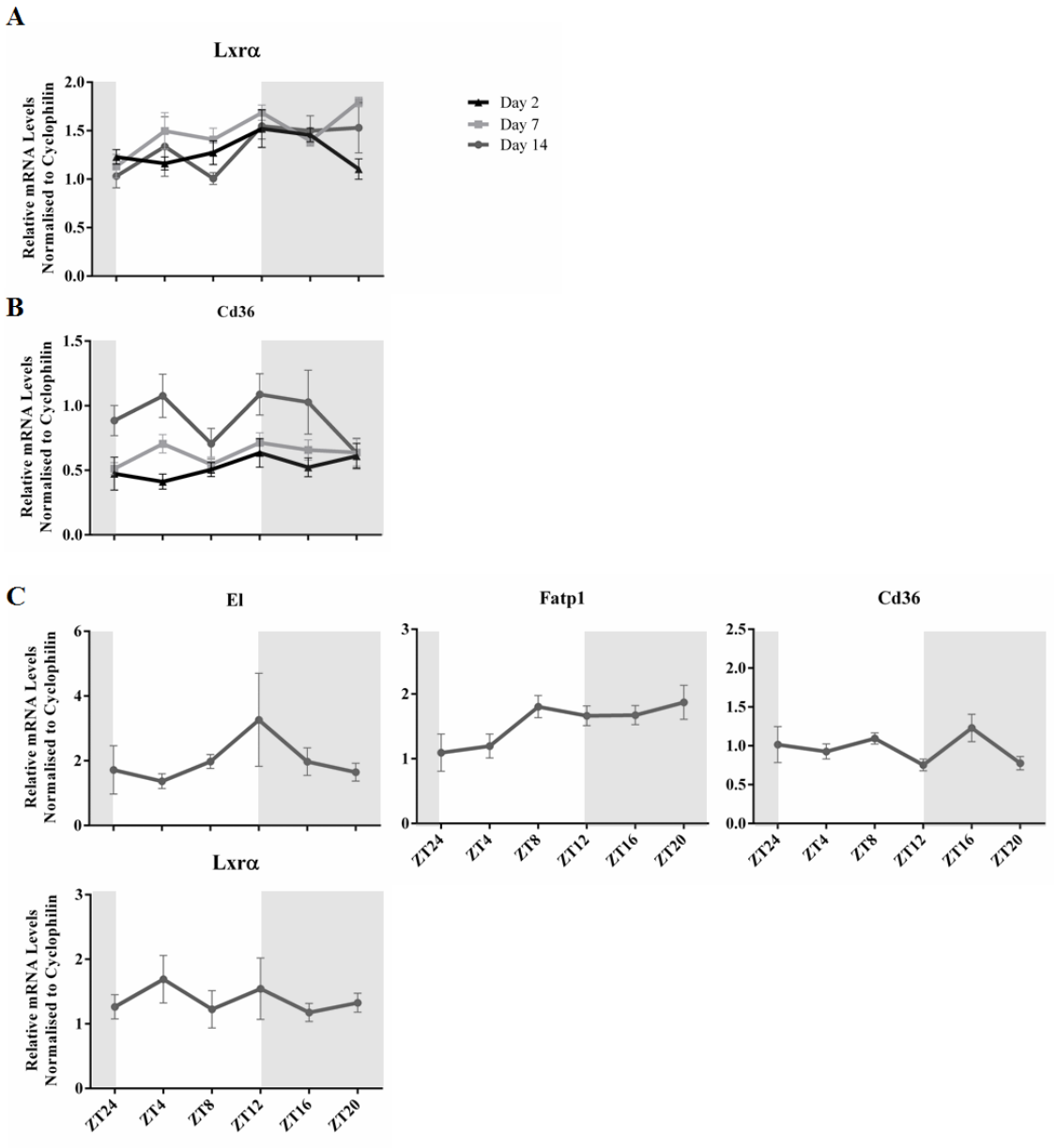
Appendix I

Supplementary Table 1: Mouse quantitative RT-PCR primer sequences

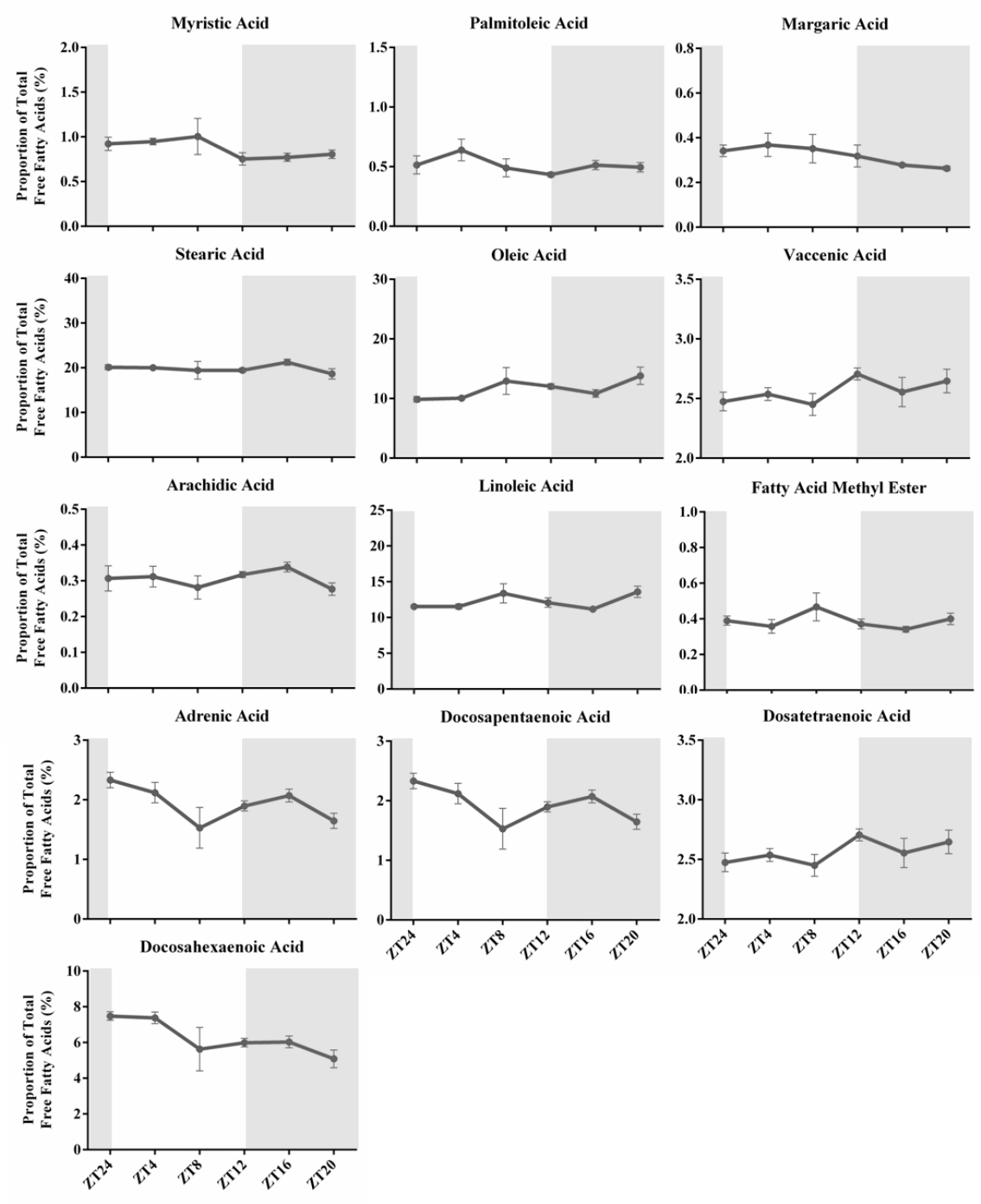
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Abca1	TCCTCATCCTCGTCATTCAAA	GGACTTGGTAGGACGGAACCT
Abcg1	GCTGTGCGTTTTGTGCTGTT	TGCAGCTCCAATCAGTAGTCCTAA
Abcg5	TCAATGAGTTTTACGGCCTGAA	GCACATCGGGTGATTTAGCA
Abcg8	TGCCCACCTTCCACATGTC	ATGAAGCCGGCAGTAAGGTAGA
Acat	ATATGAGCAAGGCTCCTCAC	GGTAGTTGTGAAAGGCATCTG
Acc1	GGCCAGTGCTATGCTGAGAT	AGGGTCAAGTGCTGCTCCA
Ap2	GATGCCTTTGTGGGAACCTG	GAATTCCACGCCCAGTTTGA
Arl7	TGAATGGACGTCTGGGTTGAG	AATCATCGCTTTGGTTCGTTG T
Bmal1	CCAAGAAAGTATGGACACAGACA	GCATTCTTGATCCTTCCTTGGT
Cd36	GATGTGGAACCCATAACTGGA	GGCTTGACCAATATGTTGACC
Clock	TTGCTCCACGGGAATCCTT	GGAGGGAAAGTGCTCTGTTGTAG
Cpt1α	AGGGCTGCACTCCTGGAAG	GACGATAAGCCAGCTGGAGG
Cpt1β	AAGAAACGCCTTATTCGAATCAA	ACCTTGCAGTAGTTGGAACCAAC
Cry1	CTGGCGTGGAAGTCATCGT	CTGTCCGCCATTGAGTTCTATG
Cyclophilin	TGGAGAGCCCAAGACAGACA	TGCCGGAGTCGACAATGAT
Cyp7a1	AGCAACTAAAACAACCTGCCAGT	GTCCGGATATTCAAGGATGCA
Cyp51a1	ACGCTGCCTGGCTATTGC	TTGATCTCTCGATGGGCTCTAT
Cytochrome C	AGACAGGCCAGGCTGCTGGA	TCCTCTCCCCAGGTGATGCCTTT
Fas	CCCAGAGGCTTGTGCTGACT	CGAATGTGCTTGGCTTGGT
Fabp3	TGACCGGAAGGTCAAGTCAC	TTCCCGTCAACTAGCTCCCT
Fabp-pm	CAAAGATGCAGAAGAAGCC	CCTCTTGCAACCATTGCT
Fatp4	TTCCCTCATCCTCCTGCT	CGATGTTTCCTGCTGAGTG
Fxr	TCCGGACATTCAACCATCAC	TCACTGCACATCCCAGATCTC
Glucokinase	CCCTGAGTGGCTTACAGTTC	ACTGATGTGAGTGTTGAAGC
Glut2	GCAAACAGCCTCTCATTGAC	GAAATTAGCCCACAATACAGT
Glut4	CCACAGAAGGTGATTGAAACAG	GAAAGGAAGAGATCATGCCA
Hmgcr	TTGGCACCATGTCAGGCGTCC	AGCGACACACAGGCCGGGAA
Hsl	CTATTCAGGGACAGAGGCAG	TAGTTCCAGGAAGGAGTTGAG
Lal	TCTTCTCAAGGACATGTTTG	CAAAGCTCCTTCATGATGAC
Ldlr	CTGGTGACCGAAAACATCCAGT	AATCAACCCAATAGAGACGGCC
Lpl	TTGAGAAAGGGCTCTGCCTG	TAGGGCATCTGAGAGCGAGT
Lxrα	AGGAGTGTCGACTTCGCAAA	CTCTTCTTGCCGCTTCAGTTT
Lxrβ	AAGCAGGTGCCAGGGTTCT	TGCATTCTGTCTCGTGGTTGT
Mcad	GATGCATCACCCTCG TGTAAC	AAGCCCTTTTCCCCTGAAG
Ncor	CTGTGCATGAGAAGCAGGAC	TGGTGAAGAATGAAGGCAAG
Npc1l1	CTACCAAGGGACGGACTACTG	TTCCTTCAAGAAAGCCTCCTC
Pdk4	CACATGCTCTTCGAACTCTTCAAG	TGATTGTAAGGTCTTCTTTTCCCAAG
Pepck	CTTGTCTATGAAGCCCTCAG	ATGATGATCTTGCCCTTGTG
Per2	ATGCTCGCCATCCACAAGA	GCGGAATCGAATGGGAGAAT
Pgc1α	TGAATGGGCCAAACAGAGAGA	TAAATCACACGGGGCTCTT
Pgc1β	GAGGGCTCCGGCACTTC	CGTACTTGCTTTTCCCAGATGA
Pkm2	TGGATGTTGGCAAGGCCCGA	AGGGCCATCAAGGTACAGGCACT
Pparα	ACAAGGCCTCAGGGTACCA	GCCGAAAGAAGCCCTTACAG
Pparγ2	TGAGCACTTCACAAGAAATTACCA	TGCGTGGTCTTCCATCAC
Rev-erbα	GGGCACAAGCAACATTACCA	CACGTCCCCACACACCTTAC
Rev-erbβ	TGGGACTTTTGAGGTTTTAATGG	GTGACAGTCCGTTCCTTTGC
Rip140	AGAACGCACATCAGGTGGCA	GATGGCCAGACACCCCTTTG
Rorα	ACCGTGTCCATGGCAGAAC	TTTCCAGGTGGGATTTGGAT
Rorγ	TCTACACGGCCCTGGTTCT	ATGTTCCACTCTCCTCTTCTCTTG
Rxrα	GACATACGTGGAGGCAAACA	AGGATGACCTGGTCGTCTAG
Scd1	CCCCTGCGGATCTTCCTTAT	AGGGTCGGCGTGTGTTTCT

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Shp	CGATCCTCTTCAACCCAGATG	AGGGCTCCAAGACTTCACACA
Smrt	ATCATCCA CCACAGAGGAG	GAATTCTATGTCGGGCTTGC
Srb1	GGGAGCGTGGACCCTATGT	ACACGGTGTCGTTGTCATTGA
Srebp1c	GGAGCCATGGATTGCACATT	GGCCCGGGAAGTCACTGT
Src1	TATCTCTCCAG CCATGGTGT	CAAAGTTCCCTTGGTTGTTGC

Appendix II



Supplementary Figure 1: Temporal oscillations in the expression of metabolic genes in murine tissues during pregnancy. **A**. Fluctuations in the expression of Lxrα over a 24-hour period in mouse liver on days 2, 7 and 14 of pregnancy. **B**. Fluctuations in the expression of Cd36 over a 24-hour period in mouse skeletal muscle on days 2, 7 and 14 of pregnancy. **C**. Fluctuations in the expression of El, Fatp1, Cd36 and Lxrα over a 24-hour period in mouse placenta on day 14 of pregnancy.



Supplementary Figure 2: Oscillations in the abundance of free fatty acids in mouse placenta over a 24-hour period on day 14 of pregnancy.